# COAXIAL ELECTROSPRAY TO PRODUCE CERASOME NANOPARTICLES FOR DRUG DELIVERY

by

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# Abstract

Cerasomes, a novel organic-inorganic nanohybrid, offer the potential to replace liposomes as a morphologically superior drug delivery vehicle. Due to the surface silicate network, cerasomes have previously demonstrated enhanced stability against membrane destabilization and lipid aggregation, thus improving therapeutic effectiveness. Here we use the one step, facile, coaxial electrospraying technique to prepare deionized water encapsulated cerasome nanoparticles. We have identified the parameters necessary to repeatedly produce particles of $217 \pm 21$ nm in size, PDI = $0.19 \pm .05$. SEM images confirmed the size measurements obtained using dynamic light scattering. The electric field strength was identified as a key factor in forming a stable electrospray process. The magnitude of the flow rate did not play a major role in affecting particle size. This study has demonstrated, for this first time, that loaded cerasomes can be successfully and repeatedly prepared using the electrospray method. Incorporating anticancer drug Doxorubicin HCl into the core of cerasomes is in progress.

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# Chapter 1

# Introduction

Advances in technology are creating endless possibilities for the future of drug delivery systems in nanomedicine. Nanoparticle carriers offer many benefits over free drug administration, such as controlled drug loading, release profiles, and tissue-specific targeting, all of which amount to a reduced organ toxicity. Research into these areas is continually uncovering new mechanisms for improved particle design and drug loading mechanisms. In addition to the use of novel carrier materials and fabrication methods, surface modifications add yet another way in which particles can be tailored for improved safety and therapeutic effect. Due to high biocompatibility and particle tailorability, liposomal drug delivery systems have shown the greatest success clinically. Despite this, liposomes suffer from having a morphologically weak phospholipid bilayer membrane that readily destabilizes in the presence of acids, bases, or surfactants. In addition to premature drug release, liposomes exhibit a tendency to aggregate and fuse together through amphipillic lipid exchange. Increased particle size makes them an easy target for body clearance systems. Both destabilization and fusion ultimately result in a reduced amount of drug reaching the desired location and thus a decrease in therapeutic efficacy. As such, liposomes remain a suitable, but limited drug delivery vehicle.

The field of nanomedicine and drug delivery systems would greatly benefit from a liposome-like drug delivering nanoparticle, but with higher morphological stability. Recently, such a carrier was created. "Cerasomes" are a novel organic-inorganic hybrid consisting of a standard liposomal membrane, but with a silicate surface. It has been repeatedly demonstrated in several preparation and characterization studies that cerasomes have improved membrane stability and a slower drug release profile than liposomes. The only attempts at creating and drug loading cerasomes have used the traditional sol-gel preparation technique. While effective, this method is a lengthy, multi-step process that produces polydisperse particles with low encapsulation efficiency and less than ideal drug loading capacity. Recently, coaxial electrohydrodynamic spraying ("electrospraying") has emerged as a simple, one-step, technique for creating core-shell nanoparticles. Published electrospray literature has demonstrated advantages of the higher encapsulation efficiency and drug loading than standard methods using various types of shell materials. However, creation of core-shell cerasome vesicles by coaxial electrospraying has not been reported thus far. For the first time, this study combines the morphological stability of cerasomes lipids with the highly effective electrospraying preparation method to produce nano-sized core-shell cerasome particles. Successful

demonstration of the ability to do so will set the stage for future cerasome drug loading studies using coaxial electrospraying.

## 1.1 Specific Aims

The goal of this study is demonstrate that core-shell vesicles from cerasome-forming organotrialkoxysilane lipids can be created using the electrospray method. Due to the novelty of this research, it will be necessary to experimentally identify the parameters that create a stable electrospray process. It is a specific aim of this study to produce cerasome vesicles of 100-300 nm, a range that is optimal for drug delivery vehicles. It is a subsequent aim for these particles to have a narrow size distribution. This will be defined as a polydispersity index (PDI) less than 0.2, measured using dynamic light scattering. Upon accomplishing these aims, it is a long range goal of this study to encapsulate anti-cancer drug Doxorubicin into the core of cerasome particles. For electrospraying to become a preferred method of producing doxorubicin loaded cerasomes, it will need to demonstrate higher encapsulation efficiency with higher drug loading content than achievable using the standard preparation techniques. Additionally, doxorubicin loaded cerasomes should be shown to have improved membrane stability, slower drug release profile, and improved therapeutic effect over PEGylated liposomal doxorubicin. The drug loading and release studies are areas for future investigation.

## 1.2 Drug Delivery Systems

Drug delivery systems (DDS) are a method or process of administering a pharmaceutical compound to achieve a therapeutic effect. The *therapeutic index* is the margin between doses resulting in therapeutic efficacy (i.e. tumor cell death) and toxicity to other organs [13]. Drug delivery systems are used to increase the *therapeutic index* of a pharmaceutical compound in several ways;

(1) Carrier systems isolate the drug from the aggressive environment of the body. Varying pH or temperature conditions can degrade the drug, alter its structure, or render it biologically inactive. Protection from the in vivo environment ensures that the correct form of the drug and intended dosage reach the target location.

(2) Carriers can be modified and tailored for specific tissue targeting. Through this, DDS not only reduce toxicity to other organs, but they allow for a higher dosage of drug to be administered [6].

(3) Carrier systems increase circulation time. Free drugs generally have shorter half lives than drug loaded carriers because carriers can be formulation to avoid body clearance by the reticuloendothelium system (RES) or glomerular filtration. The RES is characterized by mononuclear phagocytic entrapment in the liver and spleen. In theory, prolonged circulation increases the probability that the carrier systems will reach the desired or targeted location [9].

(4) DDS are more effective than free drug in that they can be modified to have optimal absorption, distribution, and drug release profiles. In general, the higher the drug loading ratio of drug/lipid (wt./wt.%) and the slower the release profile, the more effective the treatment becomes [12].

Together, these four aspects of DDS greatly improve the efficacy of a treatment.

*Important Characteristics for Drug Delivery Systems*

Drug delivering systems, in the context of nanomedicine, consist of at least two components; one of which is the pharmaceutically active ingredient, the other a non-toxic carrier vehicle. By definition, drug delivering *nanoparticles* are in the range of 10-1000 nm. It is generally accepted that the primary goals for research in nano-bio-technologies of drug delivery include; more specific drug targeting and delivery, reduction in toxicity while maintaining therapeutic effects, greater safety and biocompatibility, and faster development of new safe medicines [13].

There are four critical parameters to consider when formulation a new drug delivery system; size, drug loading, encapsulation efficiency, and release profile. *Size* is a key factor in circulation time and in the biodistribution of circulating carriers, both of which factor into therapeutic efficacy. *Drug loading*, described as the drug/lipid ratio, tells how much drug is encapsulated per vesicle. This has affects in overall dosage, which relates to both toxicity and overall therapeutic efficacy. Higher *Encapsulation efficiency* is directly linked to the commercial viability because of reduction of waste of costly pharmaceuticals. As for *release profile*, the drug-loaded vesicle must exhibit drug release rates that are conducive to improvements in drug activity through decreases in toxicity or increases in efficacy. Fast release rates are as ineffective as free drug, but slow release rates may not deliver enough of the drug to have therapeutic value [18].

*Ideal Drug Delivery Vehicle*

Nanoparticles have emerged as the leading candidate for drug delivery systems in nanomedicine. At the appropriate size (100-300 nm), nanoparticles can be formulated to evade bodily clearance, promote cellular endocytosis by target tissues, and release their contents directly at the desired site. They have high surface:mass ratios, which provide a sizable platform for surface modifications or drug coating [13]. When created with a hollow core, the drugs, probes, proteins, or genes to be delivered can be encapsulated inside the particle for protection of the compound and/or reduction of toxicity to the body. Nanoparticles carriers can be made from a variety of materials, and depending on this, bear certain advantages or disadvantages for particular applications. Biological types, such as lipids (phospholipids), proteins, carbohydrates (dextran, chitosan, or gelatin) have advantage of biocompatibility and reduced toxicity, but often suffer from weak morphologies/structures. Polymers have fairly good biocompatibility with a major advantage in "tailorability" to control circulation time, degradation and drug release. Both biological and polymer types have been greatly explored as carriers in drug delivery applications. Metallic particles such as silver, iron, gold, platinum, etc., typically suffer from being the least biocompatible, and pose the greatest concern for bodily clearance

and toxicity. Metallic particles are often utilized as contrast agents for imaging, and recently in tumor thermal ablation treatments. Certain metallic particles, due to magnetic properties, can also be utilized for site-specific targeting.

## 1.3 Liposomal Drug Carriers

### 1.3.1 Liposomes

Liposomes are artificially prepared vesicles made of a phospholipid bilayer. They were first created in 1961 by Dr. Alec Bangham using what is today known as the "Bangham method." Liposomes are amphiphilic, consisting of a hydrophilic head group and hydrophobic hydrocarbon chain tail [25]. See Figure 1.1.

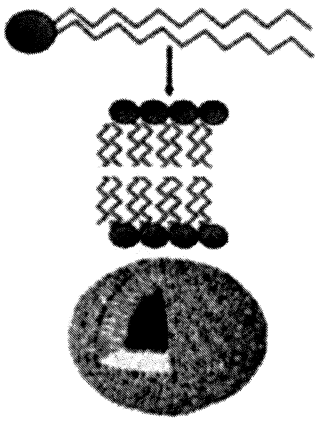


Figure 1.1: Phospholipid Liposome Vesicle [15]

In polar, aqueous solutions such as water, these phospholipids spontaneously align into spherical bilayers with the polar head groups in contact with the solution. Both the outer surface of the sphere and inner lining of the hollow core are hydrophilic surfaces. The region between these surfaces, where the carbon tails align side-by-side, constitutes the hydrophobic region. Depending on the preparation method, liposomes can exist in two main forms; unilamellar and multilamellar vesicles. Multilamellar vesicles (MLV) form spontaneously in hydrophilic solutions (Figure 1.2). Unilamellar vesicles, both small (SUV, 20-100 nm) and large (LUV, >100 nm), require high shear rate sonication (or other post-processing methods) to reduce the multiple layers into a single bilayer membrane [28].

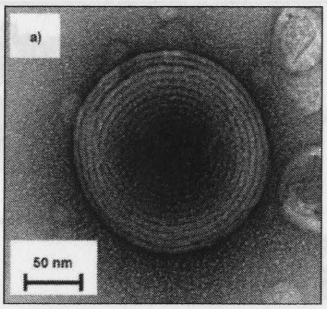



Figure 1.2: Multilamellar Vesicle [4]

Due to their similarity in structure to a cellular membrane, liposomes have for many years been used in studies as an artificial biomembrane "model." More recently, liposomes were explored for use as drug carriers, and have had great success. Liposomes, as nanosized phospholipid "fatty" structures, have the advantage of being small, flexible and biocompatible. This permits them to pass along the smallest arterioles and capillaries without causing damage or clotting [13]. Based on molecular design of the lipid components, features such as morphology, particle size, shell thickness, permeability, and other physical properties can be controlled [2]. As carriers, the hydrophilic core and a hydrophobic shell represent dual loading compartments for both hydrophobic and/or hydrophilic drugs to be loaded into the vesicles. As natural lipids, liposomes present no toxicity or clearance issues in the body [13]. This is one of the biggest problems with finding suitable carriers, and thus a major reason why liposomes have had great success. Additionally, various processing techniques can be used to control size, number of layers, surface modifications. These all have profound effects on drug loading capacity, circulation time, tissue-specific targeting, and drug release profiles. Because liposomes and target tissue cells both contain lipid membranes, liposomes are easily endocytosed into cells. This means that all of the loaded drug can be released into the desired tissue and not just in the vicinity, thus enhancing the therapeutic effect [13].

Of the numerous therapy applications explored, liposomal drug delivery systems have had the greatest success treating cancerous tumors. Tumor tissues fall victim to a phenomenon known as the leaky vasculature "enhanced permeability and retention effect" (Figure 1.3).

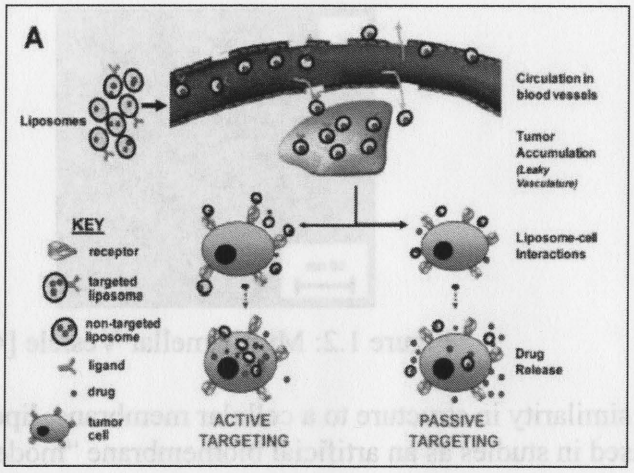



Figure 1.3: Enhanced Permeability and Retention Effect [15]

The microvascular in tumors is discontinuous, having pore sizes approximately 100-780 nm [18]. As tumor cells greatly up regulate cell division and growth, they also stimulate angiogenesis (the production of blood vessels) through VEGF and other growth factors. An increased vessel network is essential for providing oxygen and nutrients to the growing population of cells, and thus the overall viability of the tumor. Since abnormal cells are controlling this overproduction of vasculature, the resulting vessels themselves are usually defective in form and structure. They contain poorly aligned endothelial cells with wide fenestrations (or gaps) between them. They may also lack a smooth muscle layer, or contain defective endothelial/smooth muscle cells with impaired functional receptors, both of which affects its ability to dilate or constrict. Tumor tissues additionally lack effective lymphatic drainage. All of these factors lead abnormal molecular and fluid transport dynamics which is why small drugs or liposomes (100-200 nm in size) naturally accumulate in tumor tissue [26].

### 1.3.2 Success of Liposomal Drug Systems

In a drug delivery and nanoparticle review, Jong and Borm [2008] list the six biggest issues in the creation of an ideal carrier systems are;

(1) drug incorporation and release profile
(2) formulation stability and shelf life
(3) biocompatibility
(4) biodistribution and targeting
(5) functionality
(6) safe post-delivery status (i.e. clearance, uptake, etc.)

Of the various types of nanoparticles explored as drug delivery vehicles, liposomes have excelled the most in these 6 critical areas. For that reason, liposomes are the leading drug delivery systems for the systemic (intravenous) administration of drugs. According to

Puri et al [2009], there are 7 FDA-approved liposomal drugs, with 8 more in clinical trials. See Appendix A for a list of these products.

### 1.3.3 Liposomal Doxorubicin

A long range goal of this study is to encapsulate doxorubicin into cerasomes and demonstrate higher drug loading content, improved membrane stability, slower drug release, and higher encapsulation efficiency than is currently achieved with liposomal formulations. Doxorubicin was chosen for this study because it is the best known and most widely used anthracycline antibiotic for cancer therapy. It was first introduced in the 1970's and since then has become one of the most commonly used drugs for the treatment of hematological and solid tumors [18]. Due to its extreme toxicity to tissues, doxorubicin must be administered intravenously. Even so, damage to organs, particularly the heart, has limited its dosage. An approach to alleviating doxorubicin's toxicity is to use drug carriers. The advantageous properties of liposomes (as previously described) have led to their emergence as the top choice pharmaceutical carrier. In November, 1995, the FDA approved liposomal doxorubicin hydrochloride (trade name Doxil), for the first-line treatment of advanced AIDS-related Kaposi's sarcoma disease that has progressed in spite of prior combination chemotherapy, or in patients intolerant of such combination therapy [19]. Doxorubicin is an anthracycline antibiotic originally isolated from Streptomyces peucetius var. caesius. It works as an anti-tumor agent because it intercalates between the DNA strands, inhibiting topoisomerase II activity and inducing tumor cell DNA fragmentation. Additionally, liposomal doxorubicin induces expression of monocyte chemoattractant protein-1, resulting in intralesional recruitment of phagocytic cells in patients with KS [19]. As the first approved, most established and successful liposomal drug, Doxil serves as a model drug delivery system to learn from and aim at improving upon.

The primary aim of doxorubicin encapsulation in liposomes has been to decrease nonspecific organ toxicity. Liposomes are able to direct the doxorubicin away from sites with tight capillary junctions, such as the heart muscle. Instead, they distribute in areas where fenestrations or gaps exist in the vasculature (liver, spleen, and bone marrow, areas of inflammation, and *tumor tissue*). By adjusting lipid concentration and incorporating PEG-modified lipids, liposomal doxorubicin has been able to increase circulation times drastically. Increased circulation time subsequently allows more time for extravasation across the leaky endothelium of solid tumors, and thus an improved therapeutic effect [18]. Table 1.2, reproduced from Maurer et al. [2001], shows the advantage of using drug delivery vehicles (liposomal formulations of doxorubicin) over free drug.

| Formulation/trade name | Dose (mg/m²) | Plasma AUC [(mg/l).h] | $V_d$ (l) | Clearance [l/h] | TA[c] (µg/g) |
|---|---|---|---|---|---|
| Free doxorubicin[a] | 25 | 1 | 254 | 45.3 | 0.8 |
| EPC/chol (55:45)/Myocet™[b] | 25 | 19.7 | 18.8 | 23.3 | n.d. |
| HSPC/chol/PEG-DSPE (56:39:5)/ Doxil®[a] | 25 | 609 | 4.1 | 0.08 | 7.7 |

Plasma AUC: Area under the plasma clearance curve; TA: Tumour accumulation; Vd Volume of distribution

Table 1.2: Pharmacokinetic parameters and tumour accumulation of different liposomal doxorubicin formulations in humans in comparison to the drug in free form. [20]

Polyethylene glycol (PEG) is the most commonly used surface modification for both inhibiting recognition by the reticuloendothelial system and preventing agglomeration with other liposomes [13]. Both of these work to improve circulation time. The inclusion of PEGylated lipids in liposomes was one of the two most important milestones that lead to the research and development of clinically suitable liposomal formulations. The other involved an advanced method for significantly improving drug loading efficiency [15]. Since the FDA's approval of Doxil in 1995, PEGlyated liposomes have become the standardized way of preparing liposomes in both laboratory research and clinical applications. A 55:40:5 (lipid:cholesterol:PEG modified phosphatidylethanolamine) molar ratio is a standard preparation formula.

Doxil (United States) and Myocet are currently the only two liposomal doxorubicin formulations to receive clinical approval. The major difference between Doxil and Myocet is that Doxil is composed of hydrogenated soya phosphatidylcholine, cholesterol (Chol) *and PEG-modified phosphatidylethanolamine* (55:40:5 molar ratio). Myocet is composed of egg phosphatidylcholine (EPC) and Chol (55:45 molar ratio). The EPC-Cholesterol formulation tends to release drug approximately three times faster than Doxil, and has a shorter circulation time. Doxil's prolonged circulation time, due in part to the inclusion of PEG, has lead to improved efficacy of the treatment. On occasion, however, the longer circulation time has been associated with toxicities to the skin, known as Hand-Foot Syndrome [18]. In addition, the presence of large molecules of PEG on the liposomal surface may reduce interactions of liposomes with cells and hinder entry of liposomes into the tumor tissue, thereby reducing the accumulation of liposomal drug at the tumor site [12]. Although PEG has effects in reducing fusion, it does not completely eliminate it [15]. Therefore, despite Doxil's success, there remains a need for a stable, long circulating liposomal-like vesicle free of a PEGylated surface. Cerasomes, because of their surface siloxane network, may fill this void.

### 1.3.4 Limitations of Standard Liposomal Preparation Methods

One of the major issues in liposome production is the lack of a simple and effective preparation method. As previously mentioned, there a several different methods for

producing liposomes, yet no single method has emerged as the ideal way to prepare and encapsulate drug within them. The major limitations of standard technologies include polydisperse size distribution, low loading content and low encapsulation efficiency of the drug/gene. Moreover, improvement in these areas requires the use of additional lengthy and often complicated procedures.

The Bangham, also known as the "film method" or "sol-gel" technique, is the most commonly used method to prepare liposome vesicles. It is a fairly simple, but multi-step and lengthy process. First, the liposome solution is prepared by weighing out and mixing the lipid and cholesterol (and often PEG) into a glass test tube at molar ratio of 55:45 (OR 55:40:5 with PEG). Next, it is dissolved in organic solvent, i.e. chloroform, methylene chloride, or a chloroform–methanol solution (95:5 v/v). High performance liquid chromatograph (HPLC) grade solvents should be used to avoid contamination by impurities [18]. The solvent must then be removed to create a thin lipid film. It is advantageous to slowly evaporate the bulk of the solvent with a stream of nitrogen gas at a constant temperature of 37–45°C until a thick viscous lipid slurry remains. This is followed by a quick transfer to a vacuum pump (or rotary evaporator), where the sample remains under a pressure for several hours to ensure the removal of residual solvent. The dry thin film is then hydrated with the solute to be encapsulated. The lipid sample is intermittently vortexed during the hydration process to ensure maximal hydration and encapsulation. The resulting multilamellar vesicles (MLVs) are very heterogeneous and range from 500 nm to several micrometers in size. Because these sizes are both too large and too disperse to employ directly as carrier vehicles, additional processing is required to form smaller, unilamellar, and monodisperse vesicles. This is commonly done using either extrusion or ultrasonication post-processing [18].

There are several limitations or disadvantages to using the Bangham method. First, the use of an organic solvent can be troublesome. When removing the solvent, it is important that it be uniformly removed to prevent certain lipid components from crystallizing, leading to a less homogeneous lipid mixture. In addition, care must be taken in removing chloroform–methanol solvent mixtures from *cholesterol-containing* formulations. As the solvent is being evaporated, the cholesterol can precipitate in alcohols, leading to cholesterol microcrystals that will not incorporate well into the lipid bilayer after hydration. Cholesterol precipitates can also block the polycarbonate filters during the post-processing extrusion process. Aside from issues related to the *rate* of evaporation, it is extremely important that *all* of the organic solvent be evaporated. Any residual solvent has the potential to intercalate within the lipid bilayer and disrupt the ability to form stable vesicles [18]. Moreover, residual solvent may be toxic in vivo, limiting their use as drug delivery vehicles.

Another disadvantage is that the Bangham method generally results in low encapsulation efficiency and distribution of the core solution within the shell. Typically, passive trapping methods, like the one described above, do not have high encapsulation efficiencies (maximum of 80%). The maximum drug-to-lipid ratio achievable is also low

and dependent on the drugs solubility. Additionally, passive encapsulation methods require removal of the unencapsulated drug. Although this can be accomplished easily in a laboratory setting, the methods for passive encapsulation are not well suited to scaled production of pharmaceutical batches for use in clinical trials. As such, only active drug loading procedures are used to prepare liposomal doxorubicin formulations approved for clinical use. There are four principal stages involved in the preparation of *clinically used* liposomal doxorubicin:

(1) preparation of the MLVs
(2) conversion of MLVs to SUVs
(3) establishment of an ion (or pH) gradient
(4) encapsulation of doxorubicin

The key difference between passive and active trapping is the establishment of an ion gradient. The advantages with this are that drug/lipid ratios as high as 0.3/1 (wt/wt) can be achieved, and it can be used with any lipid composition that is able to form a bilayer and maintain a transmembrane ion gradient. The limitation of active loading is that it adds complicated and lengthy steps to the creation process.

Using freeze/thaw cycles within the passive loading method can improve drug loading, but this involves incorporating extra steps into the preparation process. Mayer et al. (1985) documented that upon hydration of the lipid film, the buffer solute does not equally distribute between the aqueous core of the liposome and the outside buffer. Cyclic dehydration and rehydration of the MLV lipid head groups are thought to assist in increasing the aqueous trapped volume without changing the overall vesicle size distribution. The freeze and thaw method, in general, allows efficient distribution of solute [18]. Upon the completion of this stage, the frozen MLVs can be either stored in a freezer or can be carried on to the next step. Since the Bangham process creates large, multilamellar vesicles, post-processing techniques (i.e. extrusion or ultrasonication) are still necessary to create small, unilamellar vesicles.

Reverse phase evaporation is similar in concept to the Bangham method, but is a longer and more complicated process. This method is more efficient than the Bangham "film" method because it creates unilamellar vesicles (as opposed to multilamellar) with fairly high encapsulation efficiency. One major drawback is that it is again long, multistep process that requires a complex apparatus [21]. Additionally, the particles often require post-processing size reduction, just like the Bangham method. Again, any process that uses organic solvents, removed by evaporation, run the risk of traces remaining in the final formulation that can be both damaging to particle stability and human health (if used in vivo).

*Post-processing Methods for Size and Layer Reduction*

Ultrasonication is a process by which an aqueous dispersion of phospholipids are placed in a strong bath sonicator and subject to high pressure sound waves, as a way to reduce their size and layers. The resultant liposomal particles formed are usually small unilameller vesicles (SUVs) of 25-80 nm [23]. The problems with using this method are

the polydisperse size distribution and production of particles too small to be effective drug carriers. Particles below 100 nm have a reduced core volume and are at higher risk of glomerular filtration. The optimal size drug vehicle is between 100-300 nm, based on drug loading capacity and biodistribution properties [24]. Ultrasonication is also limited in terms of what can be loaded in the core. Certain proteins, drugs, or genes may become damaged or inactive because of the aggressive nature of the high shear forces created by the ultrasound waves.

Extrusion is another method used to reduce the size, layers, and polydispersity of liposomal particles into monodisperse, small, unilamellar vesicles. In this, the liposomes are extruded under pressure through polycarbonate filters with defined pore sizes to generate a homogeneous population of smaller vesicles with a mean diameter that reflects that of the filter pore [25]. This method is also effective, but suffers from the habitual clogging of pores. A major downfall is that extruders are expensive and potentially dangerous machines that should only be operated by trained individuals.

Since none of the preparation methods described are an ideal choice for preparing liposomal structures, there is no sense in using these limited technologies for the production of a better cerasome drug vehicle. It would be advantageous to synthesize core-shell cerasome particles with a device capable of high encapsulation efficiency, high loading levels, uniform drug distribution in the core, and without high shearing forces or residual solvent issues. Additionally, it should be easy to use and scalable for high output [14]. Electrohydrodynamic spraying, discussed in detail in section 1.5, is well-suited to address these issues.

## 1.4. Cerasome

### 1.4.1 Background

Cerasomes have been labeled as novel organic-inorganic nanohybrids because they consist of an inorganic triethoxysilyl head moiety and an organic hydrophobic double-chain segment, covalently linked with stable Si-C bonds (2, Figure 1.4) [4]. These lipid *organotrialkoxysilanes* (1, Figure 1.4) are synthesized by simple condensation reactions between the three molecular units, dihexadecyamine, succinic anhydride, and 3-aminopropyltriethoxylsilane. The sol-gel/self-assembly is one of the most commonly used techniques to prepare hybrid materials, and has been the standard method for cerasome production. Using the sol-gel process (3, Figure 1.4), hydrolysis of the triethoxysilyl group converts the proamphiphile into an amphiphilic lipid. The structure is then capable of self-assembling into a liposome-like bilayer vesicle, known as a cerasome [4].

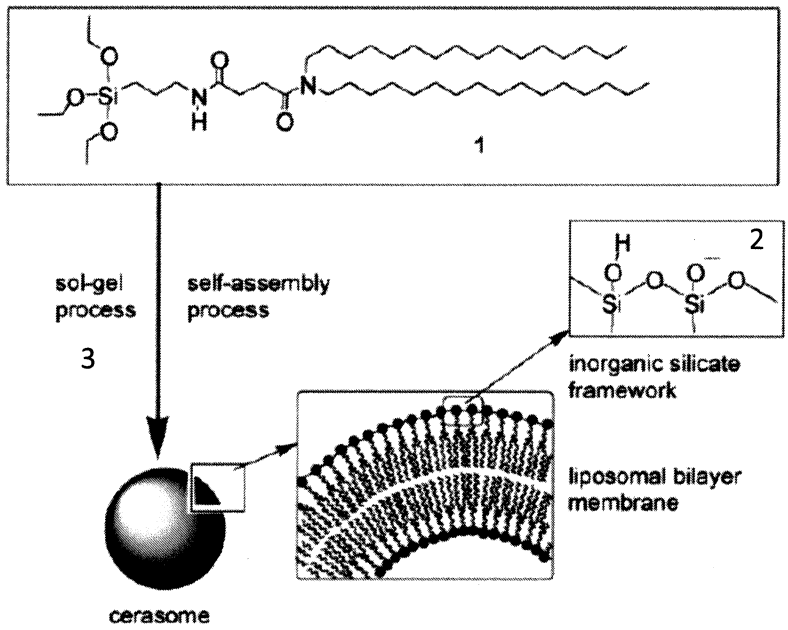



Figure 1.4: Cerasome Vesicle

Hydrolysis of the proamphiphilic compound into a bilayer forming amphiphil is a tightly regulated acid catalyzed reaction. Under acidic conditions, hydrolysis of the triethoxysilyl group precedes equally for each of the molecules in a one-by-one manner [4], providing a suitable condition for the preparation of the bilayer assembly. Under basic conditions, particular molecules are preferentially hydrolyzed while other molecules remain unreacted. Because this leads to heterogeneous hydrolysis and various reaction stages of the lipid molecule, basic conditions have been identified as unsuitable for the preparation of cerasomes. According to Katagiri et al. [2007], the conversion into amphiphilic form does not occur at neutral pH, even after vortex mixing in aqueous media. This, they claim, is likely due to the extremely slow hydrolysis of the head group.

Furthermore, the *level* of acidity factors into the successful hydrolysis of the cerasome head groups. Under very strong acidic condition (pH 1), a stable dispersion is not obtained and precipitation is observed immediately. In this case, the hydrolysis and condensation reaction were so fast that vesicular structures could not form. The acid catalyzed hydrolytic conversion from proamphiphil to amphiphil begins to proceed at pH2, yielding a well packed amphiphilic monolayer. At moderately acidic conditions of pH 3, a translucent solution characteristic of a liposomal dispersion is obtained [4]. Based on the study by Katagiri et al. [2007], it was concluded that pH 3 provides the optimal environment for cerasome formation using the sol-gel process. Figure 1.5 shows the time course for cerasome formation as evaluated from the hydrolysis of the lipids. According to this figure, it takes a minimum of 10 hours (at pH 3) to fully hydrolyze.

This data provides support for the decision to use pH3 acid ethanol to hydrolyze the lipids in our electrospray study.

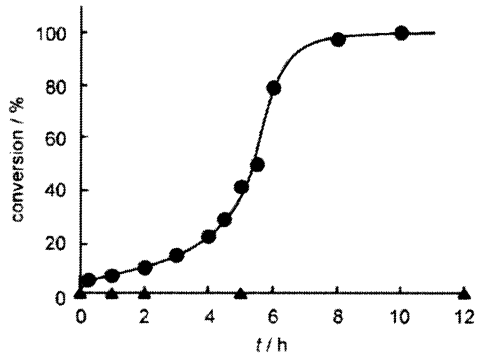



Figure 1.5: Time courses for the cerasome formation process as evaluated from the hydrolysis of the lipids (1.0 mmol $dm^3$) in D2O at pD 3 and 25°C [4]

Katagiri et al. was the first group to thoroughly characterize and document the properties of cerasome particles (Figure 1.6). In their study, they utilize the standard sol-gel/self-assembly process (followed by a short ultrasonication exposure) to create 150-300 nm. The following table presents the characteristic vesicle parameters for the cerasomes prepared. "Cerasome 1" in this figure is prepared from the same organotrialkoxysilane lipid used in our electrospray study.

| Parameter entity | Cerasome of **1** | Cerasome of **2** |
|---|---|---|
| available pH region for preparation | around 3 | 2–12 |
| reaction time for hydrolysis at pH 3 h | 10 | 2 |
| phase transition temperature[a]/°C | 10.5 | 25.7 |
| phase transition enthalpy change[a]/ kJ $mol^{-1}$ | 47.5 | 33.3 |
| hydrodynamic diameter[b]/nm | 214 | 30 |
| isoelectric point[b] | 4.3 | 12.0 |

[a] Aqueous dispersion state. [b] Ultrasonicated sample with a probe-type sonicator for 10 min at 30 W.

Figure 1.6: Cerasome Characterization by Katagiri et al. [2007]

Katagiri et al. also examine the pH dependence of zeta potential (Figure 1.7). According to their findings, cerasomes have an isoelectric point (IEP) of 4.3. Since IEP is the pH at which a particle carriers a neutral surface charge, this means cerasomes have a large negative surface charge (polyanionic) under neutral and basic conditions.

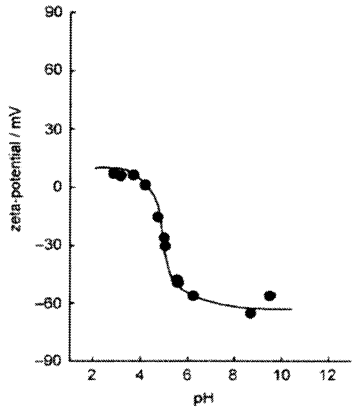



Figure 1.7: Cerasome zeta-potential vs. pH, by Katagiri et al. [2007]

Zeta potential is defined as the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed particles [29]. It is significant in that its value can be related to the stability of colloidal dispersion by indicating the degree of repulsion between adjacent, similarly charged particles. Figure 1.8 shows that a high zeta potential confers stability, whereas a low zeta potential confers a tendency of the dispersion to aggregate.

| Zeta potential [mV] | Stability behavior of the colloid |
|---|---|
| from 0 to ±5, | Rapid coagulation or flocculation |
| from ±10 to ±30 | Incipient instability |
| from ±30 to ±40 | Moderate stability |
| from ±40 to ±60 | Good stability |
| more than ±61 | Excellent stability |

Figure 1.8: Zeta potential and colloid stability [29]

According to Figure 1.7, cerasomes exhibit a zeta potential between +10 mV @ pH 3 and -70 mV @ pH 9 [1]. At neutral pH, cerasomes have a -56 mV zeta potential, indicating "good stability." This has excellent implications for the use of cerasomes as intravenous drug delivery carriers. Since the human blood is slightly alkaline (pH: 7-8), cerasomes would exhibit excellent stability.

Another benefit of using cerasome drug delivery vehicles is that they have a hydrodynamic diameter of 214 nm [4]. This fits perfectly in the range of sizes that can best avoid glomerular filtration, and RES uptake, but favor accumulate in tumor vasculature. In addition to measuring overall size, Katagiri et al. also measure the

unilaminar thickness of Si-Lipid bilayer to be approx 4 nm. Assuming 200 nm diameter particles, only 8 nm of shell means that these particles have a large hollow core suitable for significant drug loading.

Cerasomes show remarkably high morphological stability because of their surface siloxane networks, compared to weakly aligned phospholipid head groups of standard liposomes. In several studies, cerasomes have been shown to maintain their vesicular structure in the presence of membrane-solubilizing surfactants, such as Triton X-100 or ethanol. Typically, liposomes formed from unpolymerized phospholipids disassemble within 2-3 equivalents of TX-100 [1]. Note, one equivalent corresponds to a molar ratio of TX-100 to lipid of one to one. In a study by Katagiri and Caruso [2004], cerasomes remained stable passed 30 equivalents of TX-100 [1]. The later work of Katagiri et al. [2007] showed that upon 24 hours of incubation, cerasomes were stable in the presence of 36 equivalents (Figure 1.9).

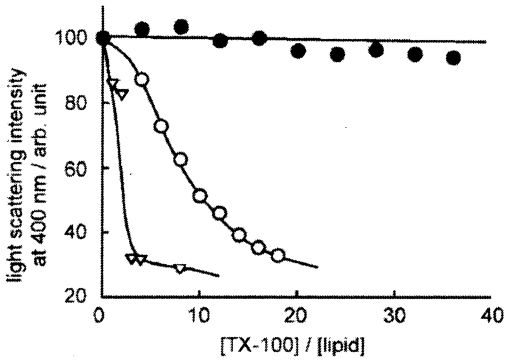



Figure 1.9: Light scattering intensities of the cerasomes and DMPC-liposome as a function of added equivalents of TX-100 at 25°C: cerasome of 1 incubated for 24 h (closed circle); cerasome as-prepared from 1, but non-incubated (open circle); DMPC-liposome (open triangle).

To provide more evidence for the advantage of cerasomes over liposomal drug vehicles, Cao et al. [2010] compared the drug release profile of cerasomes and liposomes with free drug (Figure 1.10). Here they show that cerasomes exhibit a slower drug release profile than liposomes. Free paclitaxel drug release was complete within only 10 hours. Paclitaxel loaded liposomes (PLL) had complete release by 70 hours. Paclitaxel loaded cerasomes (PLC) only released 58.2% within 120 hours. This is attributed to the formation of siloxane bonds, which result in a silica-like surface of cerasomes with a high degree of polymerization. It is believed that the siloxane networks block drug release channels and slow the rate of release. This slower drug release is beneficial for drug carriers so that the treatment can be administered less often and remains active for a

longer period of time. A drug release profile could be too slow, but the siloxane network is not so highly developed for this to happen [12].

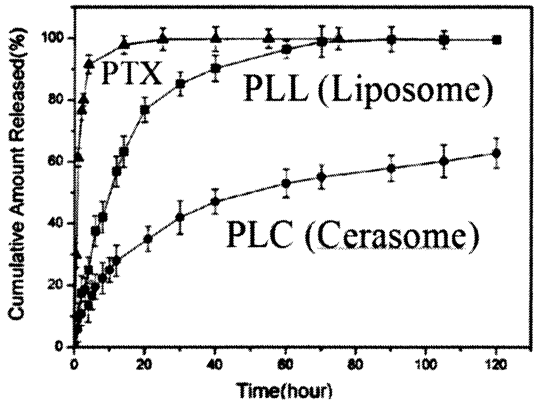



Figure 1.10: In vitro release of paclitaxel from free PTX, PLLs, and PLCs into a release medium containing 0.1% (v/v) of Tween 80 at 37 °C. Cao et al. [2010]

In addition to drug release studies, Cao et al. also test the long term stability of cerasomes in storage at 4°C. It was found that after two months in a hydrated state, cerasomes retained 88% initial drug, whereas liposomes only retained only 45%. These results provide further evidence for the advantage of a siloxane network in improving membrane stability.

This section reviews the work of several published cerasome preparation and characterization studies to provide evidence and support for the notion that cerasomes are a promising new drug system. For reasons of optimal size, excellent stability in neutral pH conditions, strong resistance against destabilizing agents (acids, bases, ethanol, TX-100), long storage stability, and a prolonged drug release profile, cerasomes are superior drug delivery vehicles than liposomes. However, despite being a promising new alternative to liposomes, there is still no ideal method for preparing cerasomes.

### 1.4.2 Cerasome Preparation Methods

To date, cerasomes have only been prepared using the standard sol-gel condensation/self-assembly method [4]. Hydrolysis of the alkoxysilyl part of the Si-lipid is performed using pH 3 acid ethanol solution. The standard molar ratio of this solution, which is also used in several cerasome studies is 1:200:19:0.03 (Si-lipid:ethanol:H2O:HCl) [1, 4, 11]. Incubation with vortex mixing for 12 hours at 25 C was identified as optimal for vesicle formation [4]. An additional 24 hour incubation time of the cerasome dispersion at room temperature is suggested to allow for complete surface siloxane network development (Equation 1.1) prior to use in any experiments [11, 12].

$$\text{Si-OCH}_2\text{CH}_3 + \text{H}_2\text{O} \rightarrow \text{Si-OH} + \text{CH}_3\text{CH}_2\text{OH}, \quad \text{followed by}$$
$$2\text{Si-OH} \rightarrow \text{Si-O-Si} + \text{H}_2\text{O}$$

Equation 1.1: Cerasome siloxane network formation [12]

Formation of siloxane network can be confirmed with FT-IR spectroscopy. A stretching band assigned to the Si-O-Si group was observed around 1100 $cm^{-1}$ in FT-IR measurements. The detectible species of the lipid oligomers have been evaluated using MALD-TOF-MS spectra.

Despite the significantly higher morphological stability against collapse or fusion with other vesicles, it has been noticed that the polydispersity of cerasomes (created using standard sol-gel methods) is quite broad. Sizes ranging from below 100 nm to several micrometers have been reported [1], which could potentially limit their use in some applications, such as drug delivery systems. In an effort to better control size and distribution, Katagiri and Caruso [2004] deposited Si-lipid membranes onto polyelectrolyte coated polystyrene solid particles to act as size templates. Upon successful formation of the lipid bilayer, the core was removed, leaving monodisperse cerasome particles. While effective, this process was a much lengthier and more complicated procedure than standard sol-gel. Preparation of cerasomes would greatly benefit from a much simpler method that can produce small particles (100-300 nm) with a narrow size distribution (<0.2 PDI), yet is also capable of high drug loading and encapsulation efficiency.

### 1.4.3 Cerasome Drug Loading

Several publications demonstrate the preparation and characterization of cerasome vesicles, but few go as far as loading them with pharmaceuticals. Cao et al. [2010], for the first time, created paclitaxel loaded cerasomes and compared the release profile and morphological stability to that of conventional PEGylated liposomes. Hydrophobic drug Paclitaxel was loaded into cerasomes according to the Bangham method in combination with sol-gel and self-assembly process. Loaded cerasomes (205.2 ±28.6 nm) had a size

and size distribution very close to that of unloaded empty cerasomes (201.3 ± 25.2 nm). This indicates that cerasome vesicle size is not significantly altered by loading drug. In addition, a PDI 0.17 ±0.03 illustrates the ability to create narrow size distribution. While the size and PDI of the particles created using the Bangham method are fairly good, the encapsulation efficiency (84 ± 4.7 %) and drug loading content (3.6 %) have room for improvement. Improvement in these areas is believed to be achievable through use of the electrospray preparation technique.

## 1.5 Electrohydrodynamic Spraying ("Electrospraying")

Cerasomes have shown superior stability over liposomes, but characteristics alone are not enough to for cerasomal drug formulations to have clinical success. They must be uniformly generated with reproducible size distributions and have a loading process that can achieve near 100% encapsulation efficiency at the desired drug-to-lipid ratio. Because the pharmaceuticals (i.e. Doxorubicin) being encapsulated are often expensive, a high encapsulation efficiency, which reduces wasting drug, is of utmost importance. The current methods used to prepare liposomal doxorubicin formulations have merely acceptable drug loading content (1/5 drug/lipid ratio → 0.2 = 20%) and good encapsulation efficiency (90-98%), but are lengthy, cumbersome processes [16, 17]. The coaxial electrospraying preparation method has been shown to match or exceed the encapsulation efficiency of standard liposomal drug formulation techniques. The big advantage, however, is in the speed and simplicity at which electrospraying can create loaded particles.

In the electrospray (ES) technique, a conducting (charged) liquid is slowly injected through an electrified capillary tube. When the electric potential between the liquid and a ground rises, a meniscus at the capillary tip develops a conical shape, commonly referred to as the Taylor cone [6].

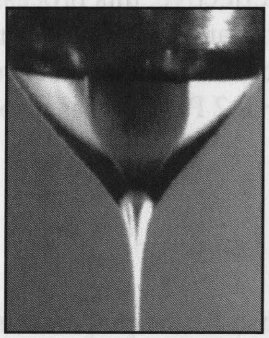


Figure 1.11: Stable Taylor cone

When electrical forces overcome the forces of surface tension of the liquid emerging from the tip of the cone, it fragments to form an aerosol spray of monodisperse, micro/nano-sized particles. The solution solvent (if one is used) evaporates before

reaching the target, and dried particles can be collection [8]. The two most important parameters involved in obtaining a stable Taylor are cone are flow rate of the solutions and strength of the electric field (created by an applied voltage).

### 1.5.1 Important Parameters

Electrospraying evolved from the process of electrospinning, a technique used to create a thin mat of fibers for scaffolds applicable in tissue engineering. Despite a nearly identical apparatus, electrospraying (into particles) is made possible through the adjustment of various parameters, namely flow rate and applied voltage. There is a specific range of voltages and flow rates where electrospraying is stable (i.e. structured Taylor cone) [14], which directly depends on the physical properties of the liquids being sprayed (i.e. conductivity, viscosity and surface tension) [6]. Of these, viscosity is the biggest player in going from thread (electrospinning) to droplet (electrospraying) formation [7]. As viscosity increases, it is thought to directly increase resistance of the solution to be separated into droplets, so it is therefore more likely to draw into a micro-thread than spray [8].

In addition to viscosity, solution conductivity is an important factor in permitting the electrospray process. To obtain a structured Taylor cone, the electrohydrodynamic forces (EHD) must act on at least one of the liquids. Any liquid without conductivity cannot be sprayed on its own, because the lack of surface charge density prevents formation of a steady Taylor cone [6]. The only way insulator-type materials can be electrosprayed is by artificially increasing their electrical conductivity via additives. Zhang and Kawakami [2010] electrosprayed solid chitosan particles and concluded that by decreasing solution conductivity, particle size increases. Xie and Wang [2007] arrived at the same conclusion when electrospraying PLGA to encapsulate BSA. Therefore, it appears consistent that by increasing conductivity and/or decreasing viscosity, particle size can be reduced [8]. Flow rate is universally mentioned as an important parameter in controlling size. Specifically, size will increase or decrease proportionally with flow rate. Other studies [9, 14] have not only verified this, but consider flow rate to be the *dominant* factor in determining particle size. For droplets emitted from a stable Taylor cone, Chakraborty et al.[2009] describe the following relationship between various parameters and particle size:

$$d = \alpha \frac{Q^{1/2} {\varepsilon_0}^{1/6} {\rho_1}^{1/6}}{{\sigma_1}^{1/6} {\gamma_1}^{1/6}}$$

Equation 1.2: Electrospray parameters on particle diameter

Where d: droplet diameter, Q: volume flow rate, $\varepsilon_0$: permittivity of free space, $\rho_1$: liquid density, $\sigma_1$: liquid surface tension, $\gamma_1$: liquid bulk conductivity, and $\alpha$: a coefficient depending on liquid permittivity.

To summarize the major parameters and their effective on droplet size; a reduction in particle size can be achieved be a decreasing flow rate, decreasing viscosity, and/or increasing conductivity. Figure 1.12 is a diagram reproduced from Chakraborty et al. [2009] depicting the influence of all electrospray parameters on particle (or fiber) diameter:

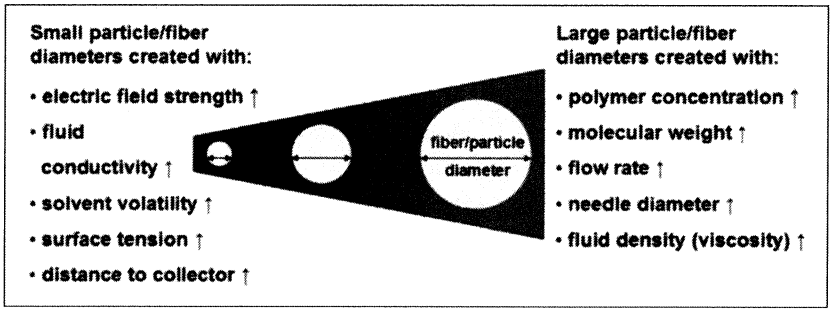



Figure 1.12: Electrospray parameters on particle diameter [14]

### 1.5.2 Drug Loading Via Coaxial Electrospraying

In 2002, Loscertales et al. first introduced the concept of *co-axial electrospraying*. In this, they aligned a smaller needle concentrically within a larger needle [6]. By doing so, they demonstrated the ability to create core-shell micro/nano particles that encapsulated the inner solution. This work sparked great interest and intense research in the field of electrospraying in nanomedicine, particularly for drug delivery systems.

There are several advantages of electrospraying over other commonly used particle preparation techniques (Bangham "film", emulsion, extrusion, ultrasonication, reverse-phase evaporation, etc.). First, this technique is flexible because it can be used to process a variety of inorganic, organic, or polymeric materials [5]. Because it is commonly used in biological applications (electrospray ionization mass spectrometry, EI-MS), fragile biological materials such bovine serum albumin (BSA), insulin, DNA, and cells have been sprayed without change or damage to their biological activity [3]. As a technique for drug delivery systems, encapsulating pharmaceuticals without harming or disrupting their biological properties is a key advantage of electrospraying. Other advantages of electrospraying include; high encapsulation efficiency, high drug loading content, and uniform distribution in the core. Furthermore, this process does not leave residual solvent, and it is easily scalable for high output [14]. All of these features make electrospraying a promising preparation method in both the laboratory and clinical setting.

Recently, Lee et al. [2010] described the use of coaxial *tri-capillary* electrospraying for multi-drug encapsulation. Here, they incorporated two drugs (Budesonide and EGCG) into a biodegradable PLGA polymer shell with near 100% encapsulation efficiency. The particles demonstrated sequential release of the two drugs. Even more, control over the core and shell flow rates allowed for precise tailoring of shell thickness (Figure 1.13), which directly relates to drug release rate.

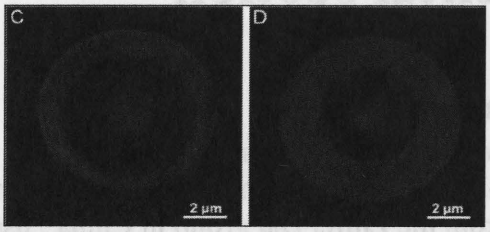



Figure 1.13: Multidrug encapsulation via coaxial electrospray [5]

The idea of a multi-drug, controlled release system has profound implications for the field of drug delivery systems. Moreover, the ability to prepare them using the most simplistic and least complicated technique to date adds merit to the process of electrospraying.

After a compilation and review of literature available on electrospraying, it is safe to conclude that this technique is capable of producing 100-200 nm sized core-shell particles with 90-98% encapsulation efficiency, and higher drug/carrier ratios than is possible with other methods. Biodegradable polymers PLGA and PLLA are the most commonly reported materials used in electrospraying. Liposomes have also occasionally been electrosprayed with success [3]. The goal of this study is to, for the first time, use the coaxial electrospray process to produce core-shell cerasomes.

# Chapter 2

# Experimental section

## 2.1 Materials

- Pure cerasome organoalkoxysilane, N-[N-(3-triethoxysilyl)propylsuccinamoyl]dihexadecylamine, MW 769.6854, was obtained from the Zhifei Dai group in China : used as drug carrier vehicle shell
- Doxorubicin hydrochloride [DXR. HCl. 14-Hydroxydaunomycin. HCl, Adriamycin. HCl, MW = 580.0, ≥95% (TLC) purity] purchased from Enzo Life Sciences via VWR.com: Anti-cancer drug
- *Actoplan* High Voltage Power Supply (Model: P030HP1M), Easton, Pa.: used to create the electric field.
- Razel syringe pump (Model R99): used for administering fluids to the needle tip to be sprayed.
- Hotplate Stirrer (Model H400-HS), Biomega Research Products Inc.: used as the base on which grounded aluminum foil and the collection dish were placed for all experiments.
- 60 x 15 mm tissue culture dishes, polystyrene nonpyrogenic. VWR: Collection dishes for sprayed particles
- *Plastibrand* disposable cuvettes (1,5 mL semimicro PMMA – 12,5x12,5x45 mm), purchased from UA stores: used for DLS measurements
- The water used in all experiments was prepared in Millipore purification system, resistivity of 19 MOhms

## 2.2 Solution Preparation

### 2.2.1 Cerasome (15 mg/mL)

The solution used to create the shell of cerasome vesicles was synthesized by adding pure cerasome-forming organotrialkoxysilane lipids to pH3 acid ethanol at a ratio of 1:10 (mg lipid:μL acid ethanol). The solution was incubated at 25°C for 24 hours to allow for hydrolysis of the cerasome lipids before being diluted to a final concentration of 15 mg/mL (19.5 mM), using pure ethanol. This stock solution was stored at 4°C for at least 24 additional hours before being used to electrospray cerasome particles.

### 2.2.2 Doxorubicin Hydrochloride

10 mg Doxorubicin HCl was dissolved in 1 mL deionized water to create a 17.24 mM solution. 10 mg/mL is the maximum solubility of doxorubicin HCl in water. Max solubility is important to optimize the drug loading (drug/lipid) weight ratio.

## 2.3 Particle Size and Morphology Measurements

The particle size and its distribution were measured with a Malvern Zetasizer Nano System (Dynamic Light Scattering instrument) at scattering angle and wavelength settings used for *Liposomes*. Z-average (Zavg) is the mean particle diameter based on the intensity of light scattered. Polydispersity index (PDI) is a measure of the distribution of molecular mass in a given sample, and ranges from 0-1. In this study, Zavg and PDI will be our primary means of determining the size and quality of particles collected with different electrospray conditions. A Zavg of 100-300 nm, with a PDI <0.2 is desired. This indicates a sample of small particles with a narrow size distribution.

Particle morphology and size distribution were investigated by scanning electron microscopy (SEM) [Hitachi S-4800 UHR FE-SEM] at 15 kV. Samples were sputter coated with platinum.

To confirm accuracy of DLS measurements, an electrospray was conducted in which particles were collected on both an aluminum peg (for SEM) and in the usually collection dish of di-water, simultaneously. The core and shell flow rates used for this experiment were 27 and 30 μL/min, respectively. A voltage of 11.5 kV was used to create a stable Taylor cone.

## 2.4 General Experimental Design

The following experimental set-up was designed based on the standard electrospray set-up described in literature. Since electrospraying has not yet been used to produce cerasomes, no single set-up or parameter values could be adopted and implemented into this study. Rather, information and ideas were gathered from a variety of publications. A set up that best suited the conditions for this study was assembled.

### 2.4.1 Apparatus

To prepare core-shell cerasome particles, a coaxial needle arrangement was used. 15 mg/mL pure cerasome lipids were fed through a capillary tube into an outer 23 gauge needle. Milipore di-water was fed through a capillary tube into an inner 27 gauge needle. The coaxial needles project downward through an orifice in a large 25"x38"x35" (height x width x depth) acrylic chamber used to prevent air contaminants from entering the experimental zone. All experiments were conducted within the chamber. A positive voltage electrode, from the high voltage power supply, was connected to the outer needle. The ground electrode ran from the power supply into the chamber through a small hole at the base and was connected to both the aluminum foil and ring electrode (see Figure 2.1, 2.2). The electric field between the positive and ground electrode connections causes the liquid meniscus emerging from the tip of the co-axial needles to draw into a conical shape, known as a Taylor cone. When the electrical forces are strong enough to overcome the surface tension in the liquid at the tip of the Taylor cone, the charged jet breaks into an aerosol spray of fine micro/nanosized particles [3, 14]. The di-water encapsulated cerasome particles were collected 11 cm below the needle tip in 6 cm diameter tissue culture dish containing 6 mL di-water. The collection dish sits directly on a 6 cm diameter circular sheet of grounded aluminum foil. A 6 cm diameter ring electrode was also grounded and placed approximately 5 cm below the tip of the needles. The idea for using a grounded ring electrode was adopted from Wu et al. [2009] to aid in directing the sprayed particles into the collection dish. A hot plate stirrer was used as the base for this set-up. A stir bar in the collection dish of di-water was used to continuously mix the solution as cerasome particles were collected. This design was intended to uniformly disperse the particles as they landed, and prevent a layer of cerasomes from accumulating at the surface. The stir bar speed was set to 3 in the range of 0-10.

Processing parameters, unless otherwise stated, were as follows: applied voltage, 11 ± 1 kV; Inner needle, 27 gauge; Outer needle, 23 gauge; Core solution flow rate, 18 μL/min; Shell solution flow rate, 20 μL/min; distance between nozzle and collection dish, H= 11 cm; distance between nozzle and ring electrode, 5 cm.

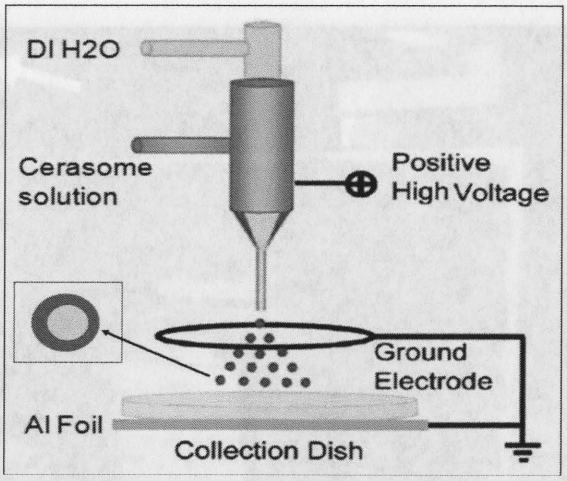



Figure 2.1: Coaxial electrospray apparatus, reproduced and modified from Wu et al. [2003]

Figure 2.2: Photograph actual coaxial needles, ring electrode, and aluminum foil

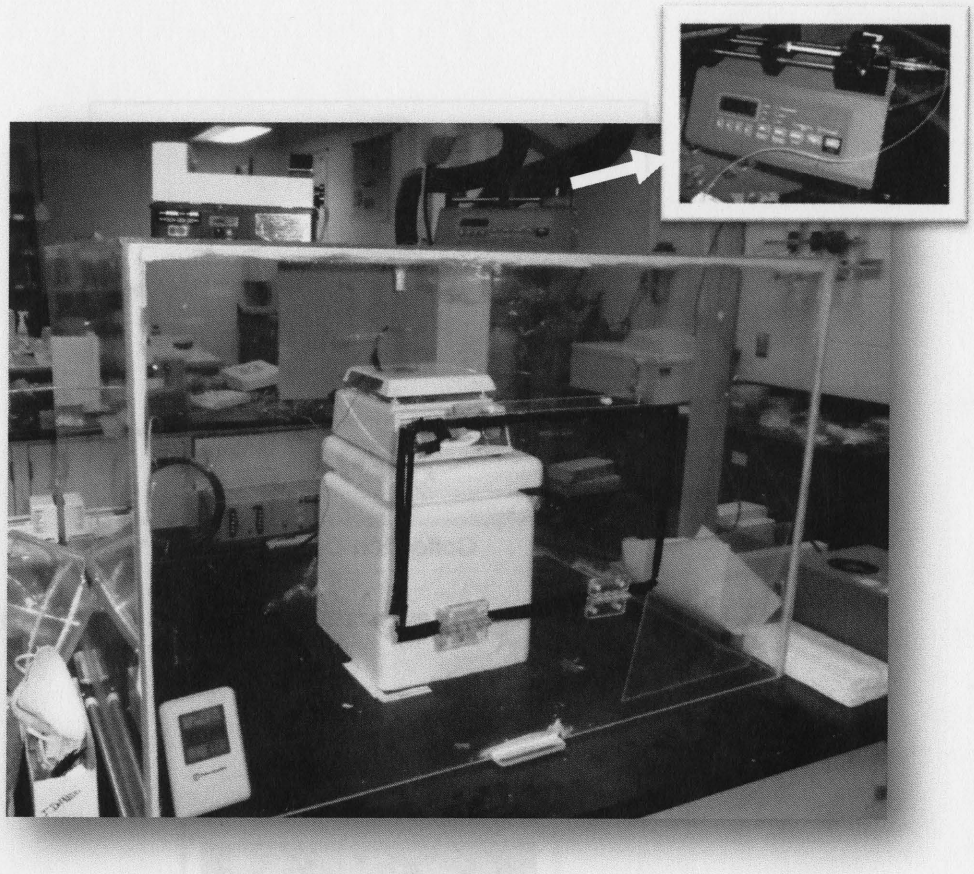


Figure 2.3: Photograph of electrospray chamber and set-up

For dynamic light scattering (DLS) size and distribution analysis, cerasomes particles were collected in a tissue culture dish filled with 6 mL di-water. For scanning electron microscopy (SEM) analysis, cerasome particles were collected directly onto aluminum pegs. A bridge of non-conducting material, placed above the di-water collection dish, was used to hold an aluminum peg. The collection dish and aluminum peg both collected particles simultaneously so that a DLS sample could be compared with the SEM images for size and distribution accuracy.

## 2.5 Methodology

### 2.5.1 Project Scope

The goal of this research is to determine the parameters required to electrospray cerasomes 100-300 nm in diameter with PDI<0.2. The novelty of this work presents opportunity to break new ground and make significant contributions to the field of nanoparticle drug delivery systems. On the other hand, it also makes the beginning phase difficult. There has yet to be published literature combining the electrospray method of creating core-shell particles with the newly synthesized organic-inorganic cerasome lipids. Therefore, the first aim of this study is to answer the question, "Can cerasomes

particles be created using the coaxial electrospray method?" Assuming they can, the next aim of this study is to determine the parameters necessary to do so. The closest related work that can assist in getting started is literature on electrospraying of liposomes [3], and publications on the basic properties of cerasomes [1, 2, 4, 11]. Although helpful in identifying a starting point, the parameters that permit the electrospraying of cerasomes (100-300 nm, PDI < 0.2) are highly dependent on solution properties, and will need to be identified through experimentation.

Due to the large number of electrospray parameter variables, it is beyond the scope of this work to examine them all. For this study, several variables were set constant. As the first aim is to demonstrate that cerasomes can be electrosprayed, experiments were created and performed with the intention of finding parameters that will accomplish this goal. The analysis of variables only went as far as being able to meet the specific aims. It is not the intention, nor a goal of this project to demonstrate and quantify the significance that *all* parameter variables have on the electrospraying process. Answering those types of questions will be undoubtedly beneficial in the precise *tailoring* of particle size, improving drug loading capabilities, and increasing efficiency. As such, that work constitutes an important and necessary set of future experiments that can build upon the groundwork laid in this study.

### 2.5.2 Parameter Constants

*Cerasome Concentration*
A concentration of 15 mg/mL was used for all experiments. This would ensure that the lipid concentration, which has affects in particle uniformity and morphology, would not play a variable role in this study.

*Needle Diameter and Spray Distance*
Co-axial needle diameters and spray distance (needle to ground) are two parameters that have been assessed in previous studies. It was determined that neither needle diameter nor spray distance play a significant role in controlling particle size [8]. Based on these findings, needle diameter and spray distance were held constant for all experiments. The co-axial needles used were 27 gauge (inner needle) and 23 gauge (outer needle). The perpendicular distance from needle tip to grounded aluminum foil was 11 cm. The grounded ring electrode was used approximately half way in between the needle and grounded aluminum foil (≈5 cm below the needle tip).

*Core Solution*
With expectations of running dozens of experiments so that parameters could be adjusted and fine tuned, it was important to use a core solution that is both readily available and inexpensive. Furthermore, with a long term goal of eventually incorporating doxorubicin into the cerasomes, it was equally important to consider a core solution that is a solvent for doxorubicin. The two important factors for choosing a solvent include cytotoxicity

and solubility. As the largest part (by volume) of the core solution, it is essential that this solvent have no adverse/toxic side effects to the body. Additionally, as a mechanism to improve the drug loading capacity, high solubility is important. Doxorubicin hydrochloride is highly soluble in dimethyl sulfoxide (DMSO, 100 mg/mL), poorly in ethanol, and fairly soluble in water (10 mg/ml). Of the three, ethanol is least soluble and least optimal for in vivo use. Despite lower solubility than DMSO, the availability and cost effectiveness of using di-water makes it the optimal choice for this study.

### 2.5.3 Effect of Electrical Field Strength (Voltage)

According to literature, the two most important parameters in electrospraying are flow rate and electric field strength (applied voltage). Because electrospraying of cerasomes has not been previously attempted, a lack of reference literature made it difficult to find an appropriate starting value for each. Various electrospray sources were compiled to create a possible range of values that may work, based on successful spraying of other materials. The operable range was found to be 5-50 μL/min flow rate and 3-20 kV voltage.

To begin experimentation, the effect of voltage on particle size and uniformity was determined by keeping flow rate constant. A publication by Chakraborty et al. provides an equation for the factors that determine particle diameter (Equation 1.2). In this, size is directly proportional to flow rate. Thus, in an attempt to produce smaller particles, the flow rate value chosen for this experiment was 10 μL/min. Particle samples were collected at four different voltages; 11 kV, 13 kV, 15 kV, and 17 kV. Each collection was sampled twice and each sample was scanned twice using DLS, thus totaling 4 scans per collection of particles. Results are expressed as the mean Zavg and PDI, ± standard deviation (n=4).

### 2.5.4 Effect of Flow Rate

To determine the effect of flow rate alone on particle size, voltage was held constant at 15 kV while adjustments were made to the speed at which the core and shell solutions were administered. Based on results of the varying voltage experiment, it was decided to use flow rates of 10, 8 and 6 μL/min.

* For all three outer flow rates (10, 8, 6 μL/min), an inner flow equal to ½ the outer was used (5, 4, 3 μL/min, respectively). The effect of this 1:2 (core:shell) flow rate ratio was examined in a later experiment, 2.4.7.

### 2.5.5 Effect of Flow Rate (with Stable Taylor Cone)

The findings of the varying voltage (2.4.3) and varying flow rate (2.4.4) experiments presented the need to examine the effect of flow rate on particle size when each flow rate had used whatever voltage would create a stable Taylor cone. Three flow rates in the range of 5-50 were examined; 5, 20, and 40 μL/min. These represent the outer (cerasome shell solution) flow rate. Core solution (di-water) flow rates were set to 4, 18, and 36 μL/min, respectively. Before collecting particles, the voltage setting was adjusted manually until a visibly stable Taylor cone was observed. Once the voltage required to create a stable Taylor cone was found, the experiments were carried out as previously described. It was hypothesized, based on findings in literature, that particle size would increase with increasing flow rate, and vice versa. Because voltage was adjusted until a stable cone was observed, it was additionally believed that there would be no significant difference in size distribution (PDI) among the different flow rates. Three independent electrospray trials were conducted for each flow rate. Results are presented as the mean Zavg and PDI (± standard deviation), n=3. Statistical analysis of significance was based on 95% confidence interval ($p \leq 0.05$).

### 2.5.6 Down vs. Offset Collection Dish

It was observed that when voltage is not within the range of 11 ± 1 kV, the Taylor cone becomes visibly unstable and gives way to a dripping rather than sprayed solution. To separate out and collect only the electrically sprayed particles, the collection dish was moved slightly offset from directly below the coaxial needles. An assessment of the significance of this offset was done using both a straight down and offset dish together. The offset collection dish was placed on the grounded aluminum foil. It was hypothesized that the particles being sprayed under the influence of the electric field would converge through the ring electrode, towards the grounded foil, and get collected in the offset dish. Any large particles that drip from the needles when the cone is unstable would be collected in the dish directly below the needles. Based on this hypothesis, it was expected that the offset dish would contain smaller particles with a lower PDI compared to the downward dish. Size and uniformity of the particles from both collection dishes were evaluated in terms of Zavg and the polydispersivity index (PDI) obtained from DLS measurements. Statistical analysis of the significance between down and offset was based on 95% confidence interval ($p \leq .05$).

### 2.5.7 Ratio of Core:Shell Solution Flow Rate, Affect on Particle Size

It was suggested in a previous study that the ratio of inner (core) to outer (shell) flow rate not only plays a role in encapsulation efficiency, but overall particle size as well [5]. To test the affect on size, two shell (cerasome) solution flow rates were examined; 10 and 20 μL/min. For each, two different core:shell ratios were examined; 1:1 and 1:2. Statistical analysis of the results (based on Zavg) are used to determine if the difference between core and shell solution flow rates play a significant ($p \leq .05$) role in affecting particle size.

### 2.5.8 Repeatability

Once the parameters necessary to create 100-300 nm size cerasome particles with PDI <0.2 were determined, the ability to repeatedly produce them was examined. For this, six independent electrosprays (n=6) were conducted using the exact same parameters:

Flow Rate: 18, 20 μL/min (core, shell)
Voltage = 11 ± 1 kV

The size and PDI were measured using DLS. The 6 trial mean of the Zavg and PDI (± standard deviation) were calculated and used at the final particle size and distribution that this study claims to be capable of repeatedly producing.

### 2.5.9 Particle Stability

The particle stability was examined to determine if storage in collection solution (di-water) had an effect on size or distribution. The surface siloxane network was originally introduced onto lipid bilayer structures to make cerasomes more stable than liposomes towards both aggregation and membrane destabilization. It is thus expected that cerasomes are capable of maintaining their size and PDI during storage conditions. To test this, a single collected solution of sprayed cerasome particles was measured for size and PDI using DLS over the course of 2 weeks (Day 1, 2, 6, 14). Stability results are presented as change in Zavg and PDI over time.

# Chapter 3

# Results

## 3.1 Effect of Electrical Field Strength (Voltage)

By holding flow rate constant at 10 μL/min and varying voltage between 11 and 17 kV, a clear pattern between voltage Taylor cone stability emerged. At a flow rate of 10 μL/min, an 11 kV electric field produced the smallest (293 ± 7 nm) cerasome particles with lowest PDI (0.17 ± .04). As the decreased from 17 to 11 kV, the Taylor cone gradually stabilized, causing both particle size and PDI to decrease. The results from this experiment is listed in Table 3.1. Graphical representation of the results, shown in Figures 3.1 and 3.2 (Size vs Voltage and PDI vs Voltage) correlate well with one another, and with the visuals of Taylor cone formation and stability (Figure 3.3).

| | | Flow Rate = 10 microL/min | | | | | |
|---|---|---|---|---|---|---|---|
| Voltage [kV] | | Scan 1 | Scan 2 | Scan 3 | Scan 4 | Average | Stand. Dev |
| 11 | Zavg | 302.7 | 292.7 | 292.6 | 286.7 | 294 | 7 |
| | PDI | 0.117 | 0.152 | 0.19 | 0.207 | 0.17 | 0.04 |
| 13 | Zavg | 312.8 | 312.1 | 313.6 | 306.1 | 311 | 3 |
| | PDI | 0.233 | 0.271 | 0.224 | 0.229 | 0.24 | 0.02 |
| 15 | Zavg | 349.5 | 345.4 | 432.5 | 424.6 | 388 | 47 |
| | PDI | 0.431 | 0.415 | 0.472 | 0.46 | 0.44 | 0.03 |
| 17 | Zavg | 636.1 | 575.5 | 689.6 | 782.3 | 671 | 88 |
| | PDI | 0.627 | 0.609 | 0.627 | 0.71 | 0.64 | 0.05 |

Table 3.1: Varying voltage with constant flow rate

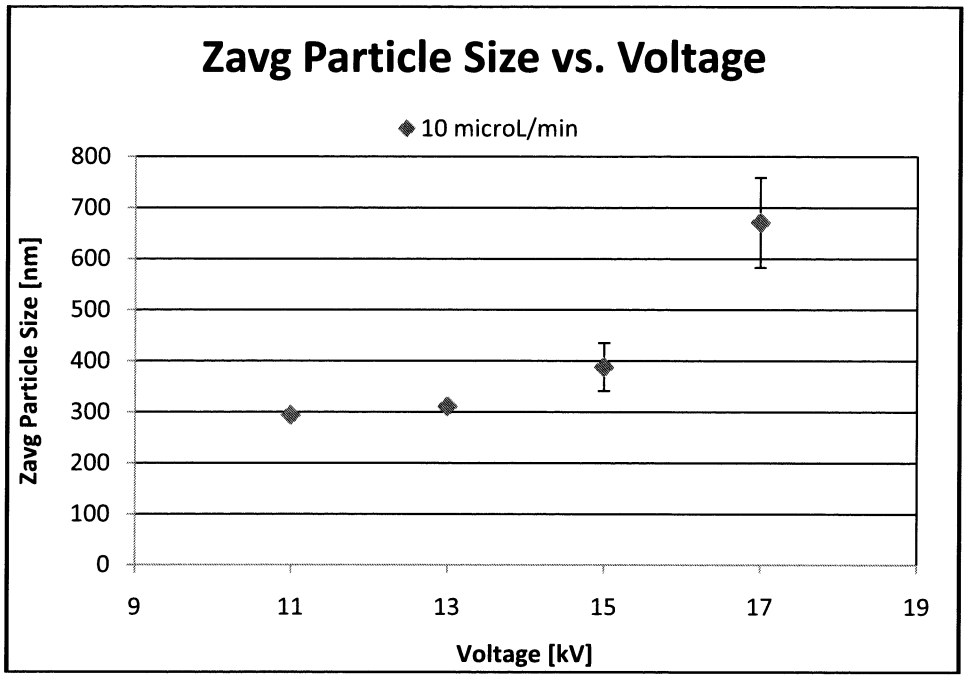



Figure 3.1: Zavg particle size vs. varying voltage (constant flow rate)

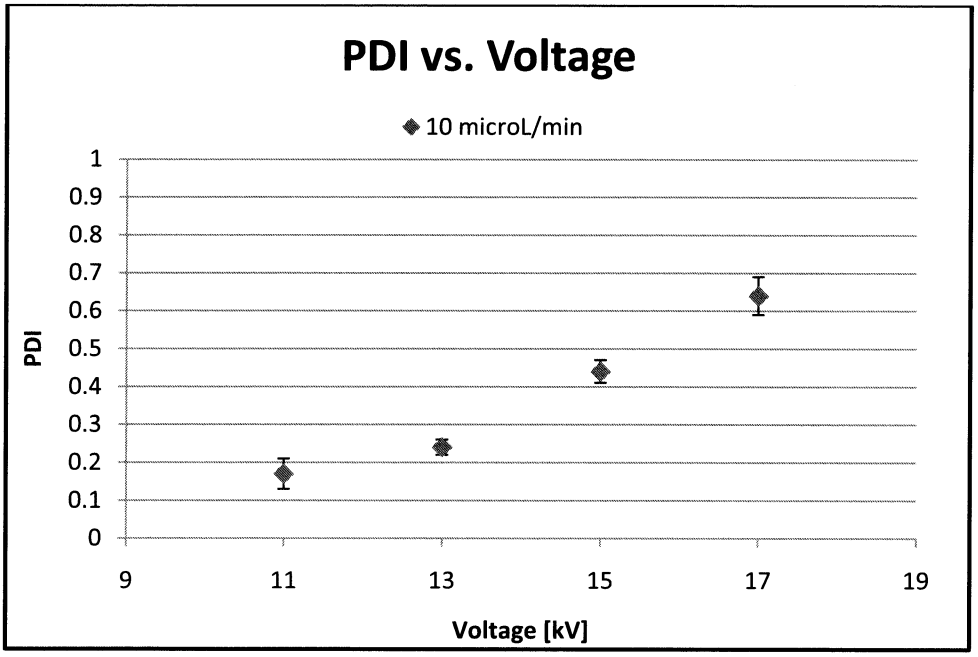



Figure 3.2: PDI vs. varying voltage (constant flow rate)

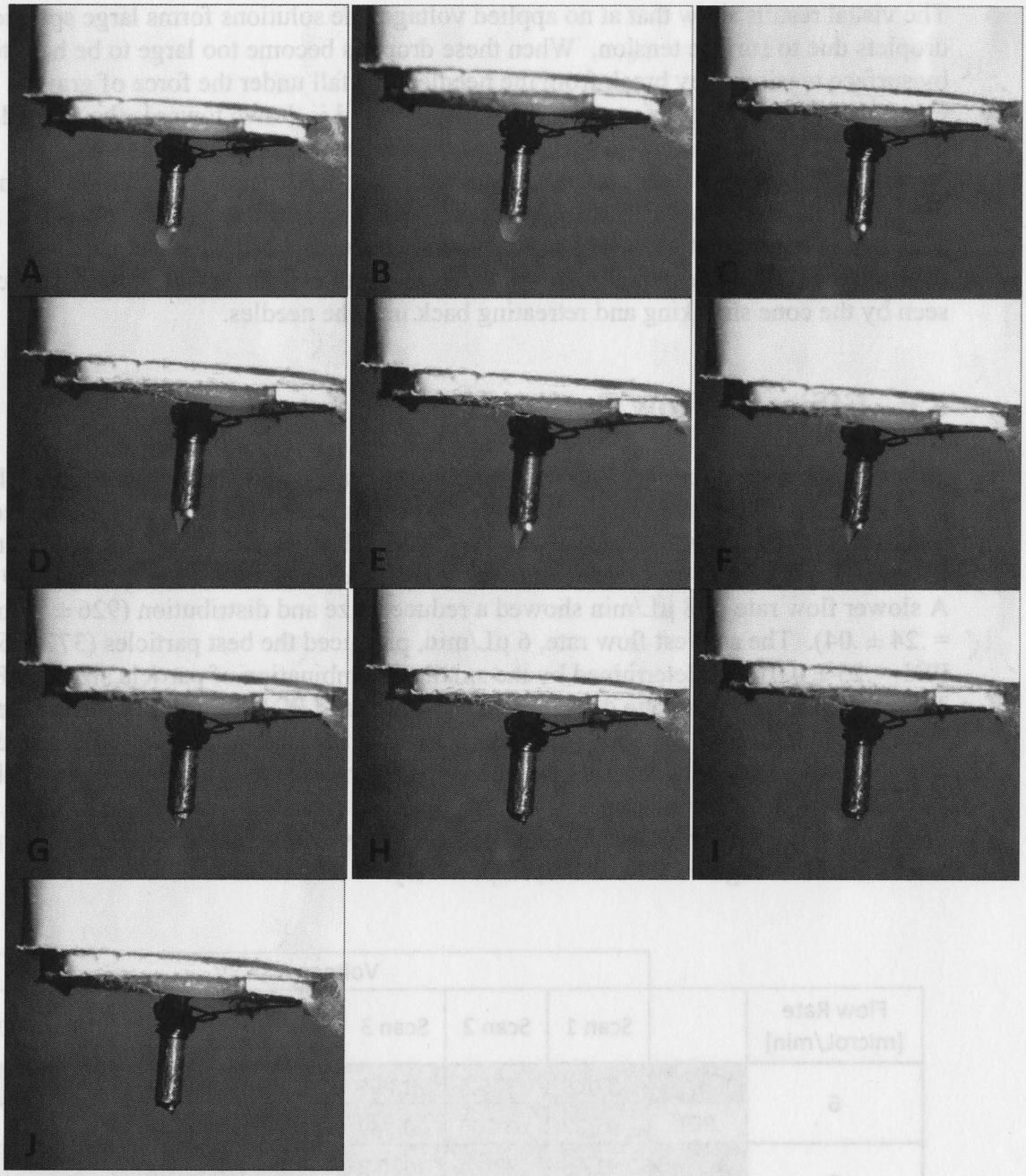



Figure 3.3 Visual effect of adjusting voltage on Taylor cone stability.
Flow rate = 10 μL/min.
Voltage [kV] = A. NO VOLTAGE, B. 7 kV, C. 8 kV, D. 9 kV, E. 10 kV, F. 11 kV, G. 12 kV, H. 13 kV, I. 14 kV, J. 15 kV.

The visual results show that at no applied voltage, the solutions forms large spherical droplets due to surface tension. When these droplets become too large to be held together by surface tension, they break from the needles and fall under the force of gravity. However, when voltage is applied, the charged liquid is drawn towards the ground. This electrical force competes with surface tension to atomize the liquid. As voltage is increased, the electric field gradually overcomes surface tension and draws the spherical droplet into a cone shape. This "Taylor cone" takes shape at approximately 9 kV, and is most stable between at 11 ± 1kV. Once voltage exceeds 12 kV, the electric field overpowers surface tension and the solution cannot draw into a stable cone. This can be seen by the cone shrinking and retreating back into the needles.

## 3.2 Effect of Flow Rate

This experiment was performed to evaluate if flow rate could also be used to stabilize the Taylor cone and improve particle quality. To do this, we began with an unstable cone, and tried to adjust flow rate to stabilize it. Using a flow rate of 10 μL/min and voltage 15 kV, particles collected were very large and polydisperse (1280 ± 42 nm, PDI = .49 ± .05). A slower flow rate of 8 μL/min showed a reduced size and distribution (926 ± 9 nm, PDI = .24 ± .04). The slowest flow rate, 6 μL/min, produced the best particles (377 ± 6 nm, PDI = .25 ± 0.01), as determined by the smallest combination of particle size and PDI. Therefore, varying flow rate did affect particle size and PDI, but the key difference is that it was not able to form a stable Taylor cone from an unstable cone (like voltage did). These experiments point towards voltage being the major factor in Taylor cone stability and thus particle size uniformity. The data obtained from this experiment is listed in Table 3.2. Graphical representation of particle size vs. flow rate and PDI vs. flow rate can be found in Figures 3.4 and 3.5, respectively.

| | | Voltage = 15 kV | | | | | |
|---|---|---|---|---|---|---|---|
| Flow Rate [microL/min] | | Scan 1 | Scan 2 | Scan 3 | Scan 4 | Average | Stand. Dev |
| 6 | Zavg | 378.5 | 375.5 | 384.5 | 371.3 | 377 | 6 |
| | PDI | 0.237 | 0.266 | 0.247 | 0.244 | 0.25 | 0.01 |
| 8 | Zavg | 917.9 | 930.4 | 920.3 | 936 | 926 | 9 |
| | PDI | 0.192 | 0.275 | 0.219 | 0.269 | 0.24 | 0.04 |
| 10 | Zavg | 1249 | 1287 | 1247 | 1337 | 1280 | 42 |
| | PDI | 0.46 | 0.503 | 0.453 | 0.553 | 0.49 | 0.05 |

Table 3.2: Varying flow rate with constant voltage

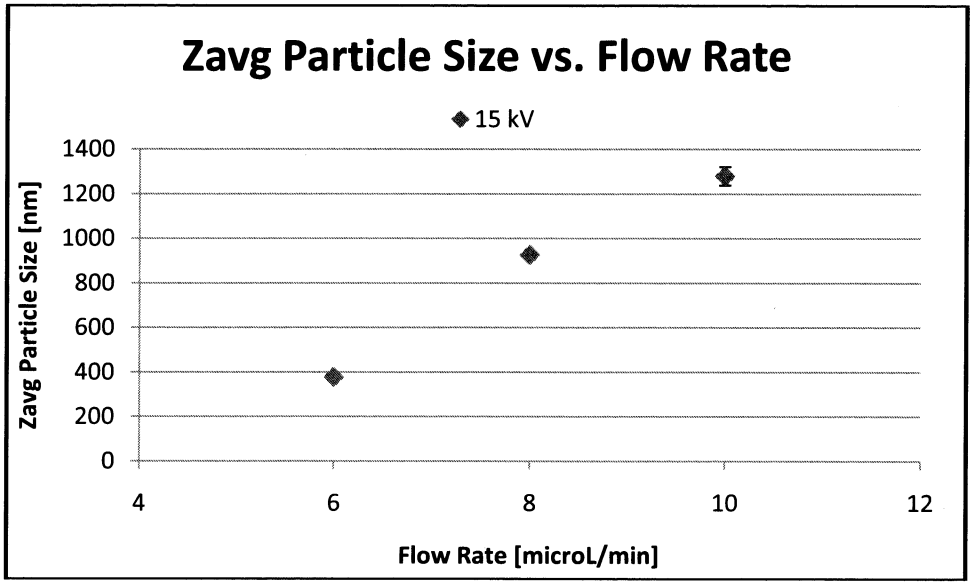



Figure 3.4: Zavg particle size vs. varying flow rate (constant voltage)

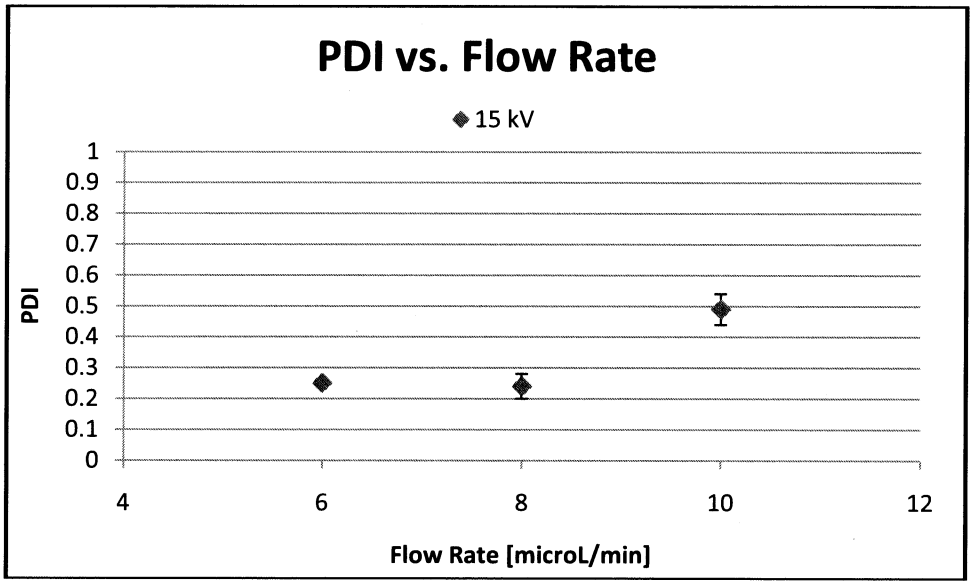



Figure 3.5: PDI vs. varying flow rate (constant voltage)

## 3.3 Effect of Flow Rate (with Stable Taylor Cone)

The results of the *varying flow rate* and *varying voltage* experiments inspired the need to test whether or not particle size can be tailored by varying flow rate, but this time with a stable Taylor cone. In this particular experiment, 5, 20, and 40 μL/min were examined. For each flow rate, the voltage value was adjusted until a stable Taylor cone emerged. Figure 3.6 shows visuals of the formation of a stable Taylor cone as voltage is manually adjusted. Data from three independent experiments were collected and listed in Table 3.3 (See Appendix B). Figure 3.7 is a graphical depiction of the data in Table 3.3. Figure 3.8 shows the differences in mean particle size (± standard deviation) created by the three flow rates. Statistical significance of the results is listed in Table 3.4.

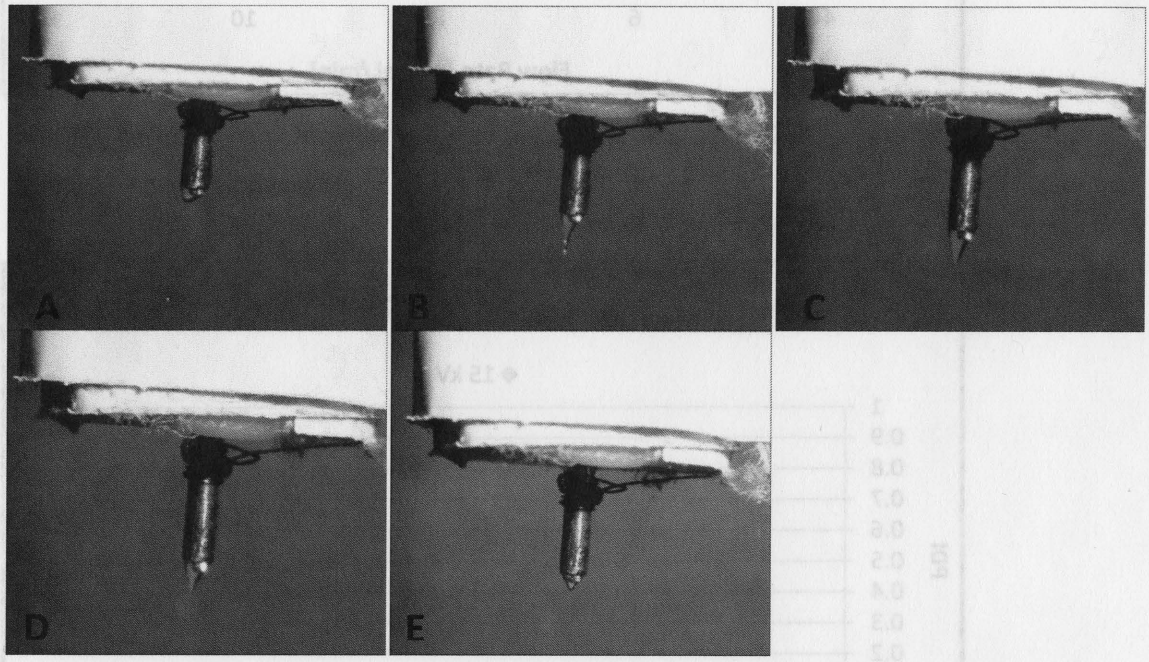



Figure 3.6: Formation of a stable Taylor cone as voltage is adjusted.
Flow rate = 20 μL/min.
Voltage [kV] = A. 8 kV, B. 9 kV, C. 10 kV, D. 11 kV, E. 12 kV

These photographs show that for 20 μL/min flow rate, voltages 11 ± 1 kV form a stable Taylor cone. Interestingly, the same hold true for all flow rates of 5 through 40 μL/min (pictures not shown).

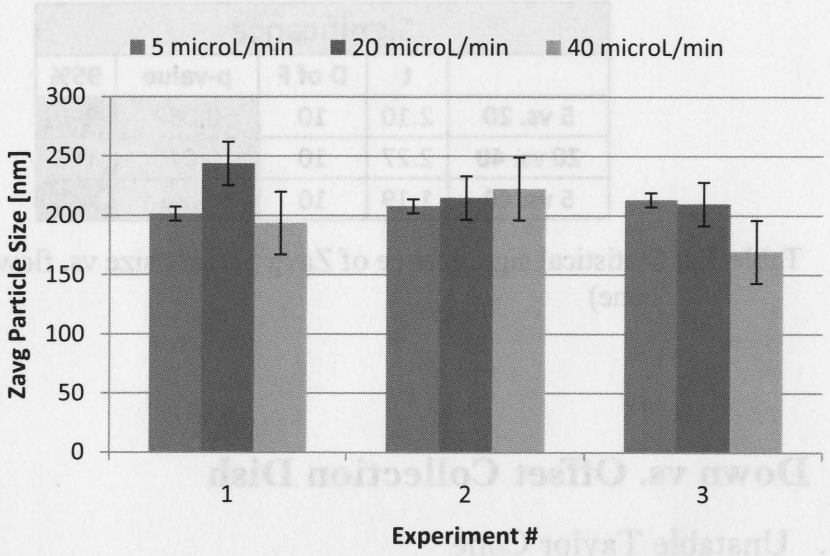



Figure 3.7: Zavg particle size vs. flow rate (stable Taylor cone)

In this experiment, voltage was adjusted to create a stable Taylor cone for each flow rate tested. It was expected, based on prior experiments, that the voltage required to make a stable cone would vary significantly for the different flow rates tested. Interestingly, 11 ± 1 kV was the required voltage for all three flow rates (5, 20, and 40 µL/min).

Because all flow rates were tested under conditions of a stable cone, each sample collected had a PDI < 0.2, which reflects a narrow size distribution of cerasome particles. This experiment provides more evidence for the importance of creating a stable Taylor cone. Despite this finding, no clear relationship between varying flow rate and particle size was observed. All three flow rates produced particles between 190-230 nm. Statistical analysis shows the lack of any significant difference.

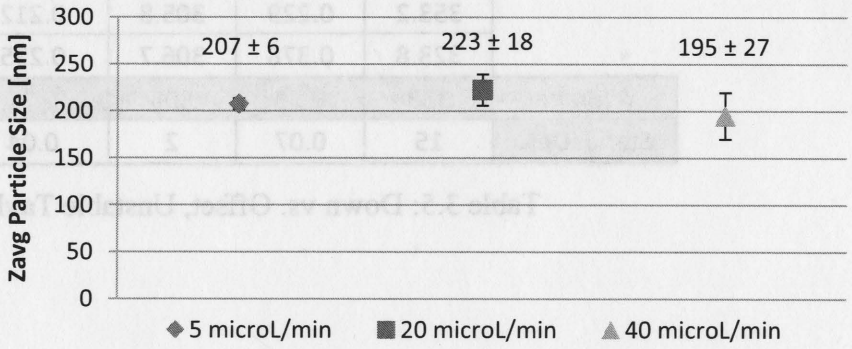



Figure 3.8: Mean (n=3) of Zavg particle size vs. flow rate (with stable Taylor cone)

| Significance | | | | |
|---|---|---|---|---|
| | t | D of F | p-value | 95% |
| 5 vs. 20 | 2.10 | 10 | 0.062 | NO |
| 20 vs. 40 | 2.27 | 10 | 0.046 | YES |
| 5 vs. 40 | 1.19 | 10 | 0.26 | NO |

Table 3.4: Statistical significance of Zavg particle size vs. flow rate (stable Taylor cone)

## 3.4 Down vs. Offset Collection Dish

### 3.4.1. Unstable Taylor Cone

When the Taylor cone is unstable, there is a visible drip from the needle tip. Therefore, in an effort to try and separate out the large droplets from nicely sprayed particles, an offset collection dish set-up was tested. An unstable Taylor cone was intentionally created using a voltage of 15 kV, well above the stable range of 11 ± 1 kV. The results of the particles collected in the down dish compared the offset dish are as follows:

| | Flow Rate = 10 µL/min Voltage= 15 kV | | | |
|---|---|---|---|---|
| | DOWN | | OFFSET | |
| | Zavg | PDI | Zavg | PDI |
| | 348.2 | 0.342 | 302.8 | 0.24 |
| | 327.3 | 0.368 | 307.3 | 0.296 |
| | 353.2 | 0.229 | 305.8 | 0.212 |
| | 323.8 | 0.378 | 306.7 | 0.235 |
| Average | 338 | 0.33 | 306 | 0.25 |
| Stand. Dev. | 15 | 0.07 | 2 | 0.04 |

Table 3.5: Down vs. Offset, Unstable Taylor cone

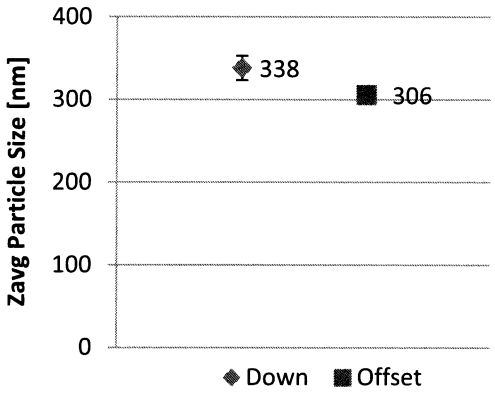



Figure 3.9: Down vs. Offset, Zavg particle size - mean and standard deviation (Unstable Taylor cone)

The offset dish collected smaller particles (306 ± 2 nm) with a narrower size distribution (PDI = 0.25 ± 0.04) than the vertical down dish (338 ± 15 nm, PDI = 0.33 ± 0.07). Statistical analysis reveals a significant (p = 0.0047) difference in the particles collected based on size (Zavg), but not significant difference (p = 0.074) based on PDI.

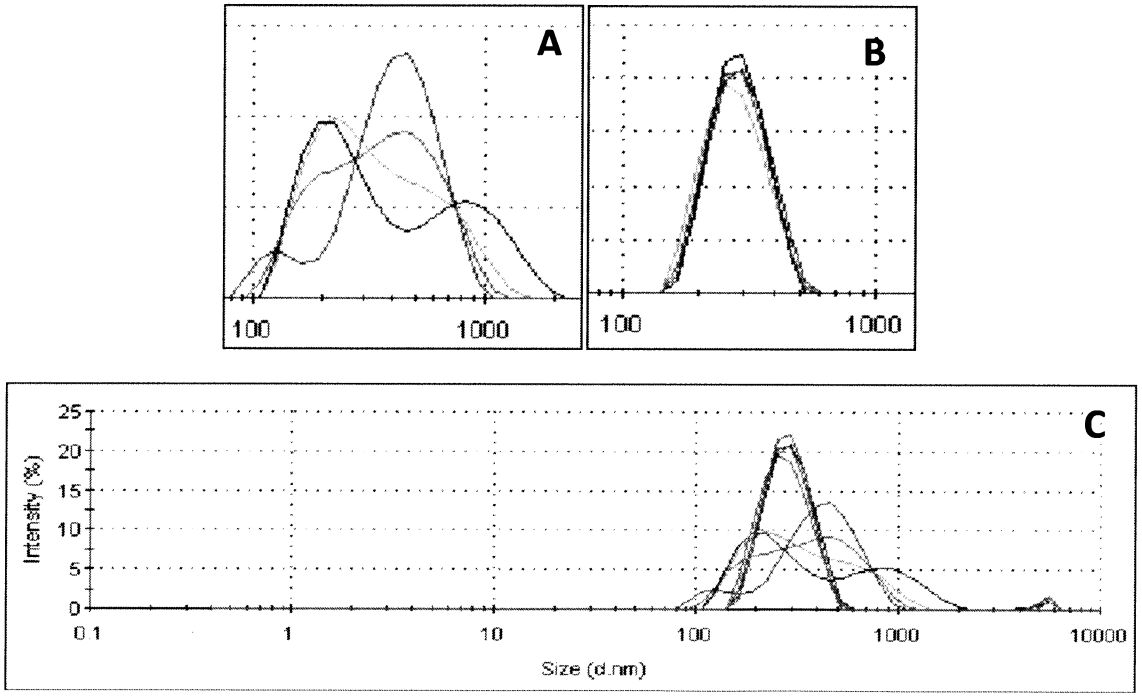



Figure 3.10: Unstable Taylor cone. DLS: Zavg size measurements of particles collected; A. straight down, B. offset, and C. shown plotted together.

### 3.4.2 Stable Taylor Cone

The same experiment was conducted to test the effectiveness of an offset dish, but this time using a stable Taylor cone.

| | Flow Rate = 20 µL/min Voltage= 11 kV | | | |
|---|---|---|---|---|
| | DOWN | | OFFSET | |
| | Zavg | PDI | Zavg | PDI |
| | 190.4 | 0.156 | 208.2 | 0.278 |
| | 198.8 | 0.167 | 217 | 0.248 |
| | 217.6 | 0.158 | 258.1 | 0.246 |
| | 217.6 | 0.137 | 263.7 | 0.287 |
| Average | 206 | 0.15 | 237 | 0.26 |
| Stand. Dev. | 14 | 0.01 | 28 | 0.02 |

Table 3.6: Down vs. Offset, Stable Taylor cone.

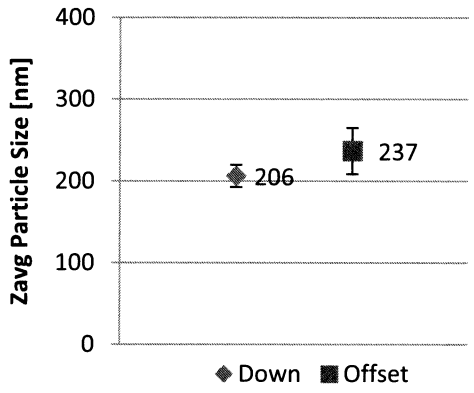



Figure 3.11: Down vs. Offset, Zavg particle size - mean and standard deviation (Stable Taylor cone)

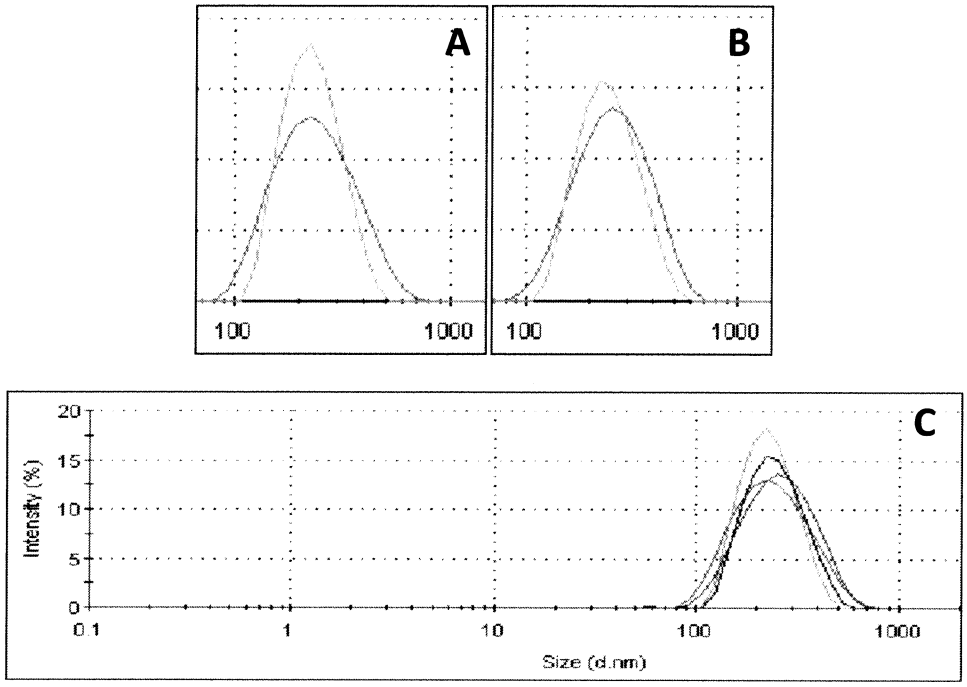



Figure 3.12: Stable Taylor cone. DLS: Zavg size measurements of particles collected; A. straight down, B. offset, and C. shown plotted together.

When an appropriate voltage is used to create a stable Taylor cone, the particles being produced have a narrow size distribution. Additionally, there is no visible transient drip from the needle tips because the electric field is of the exact strength needed to completely aerosolize the solution into nanoparticles. These results show there is no significant difference between the size of particles collected down (206 ± 14 nm) and those collected at an offset (237 ± 28 nm), $p = .098$, when the cone is stable. Interestingly, it was also found that the down dish collected smaller particles with a narrower size distribution that the offset dish ($p < 0.0001$). Taken together, this is evidence that an offset collection dish is beneficial when the Taylor cone is unstable, but has no benefit over a straight down dish when the electrospray process is stable.

## 3.5 Ratio of Core:Shell Flow Rate, Affect on Particle Size

It was assessed whether the core:shell solution flow rate ratio had any significant effect on overall particle size or distribution. Two different ratios; 1:1 and 1:2 were tested for the three different outer flow rates 10, 20, 30 μL/min. When the outer solution flow rate was 10 μL/min, data shows that there is significant difference between particles created from a 1:1 ratio (294 ± 7 nm) and those from a 1:2 ratio (307 ± 6 nm), $p = 0.025$. When the outer solution flow rate was 20 μL/min, data shows again that there is significant

difference between particles created from a 1:1 ratio (216 ± 5 nm) and those from a 1:2 ratio (177 ± 5 nm), p = 0.022. When an outer solution flow rate is 30 μL/min, there is no significant difference between particles created from a 1:1 ratio (208 ± 3 nm) and those created from a 1:2 ratio (212 ± 2 nm), p = 0.059.

| Core:Shell [μL/min] | | Scan 1 | Scan 2 | Scan 3 | Scan 4 | Average | Stand. Dev |
|---|---|---|---|---|---|---|---|
| 10:10 | Zavg | 302.7 | 292.7 | 292.6 | 286.7 | 294 | 7 |
| | PDI | 0.117 | 0.152 | 0.19 | 0.207 | 0.17 | 0.04 |
| 5:10 | Zavg | 312.9 | 313.1 | 301.4 | 302.3 | 307 | 6 |
| | PDI | 0.193 | 0.203 | 0.221 | 0.226 | 0.21 | 0.02 |
| 20:20 | Zavg | 223.6 | 211.9 | 215.6 | 213.9 | 216 | 5 |
| | PDI | 0.136 | 0.177 | 0.18 | 0.139 | 0.16 | 0.02 |
| 10:20 | Zavg | 233 | 227.4 | 224.1 | 222.8 | 227 | 5 |
| | PDI | 0.121 | 0.096 | 0.113 | 0.122 | 0.11 | 0.01 |
| 30:30 | Zavg | 209 | 204.2 | 211.5 | 206.6 | 208 | 3 |
| | PDI | 0.126 | 0.121 | 0.101 | 0.106 | 0.11 | 0.01 |
| 15:30 | Zavg | 214.4 | 210.9 | 211.9 | 210.7 | 212 | 2 |
| | PDI | 0.12 | 0.153 | 0.126 | 0.127 | 0.13 | 0.01 |

Table 3.7: Ratio of core:shell flow rate, Effect on particle size.

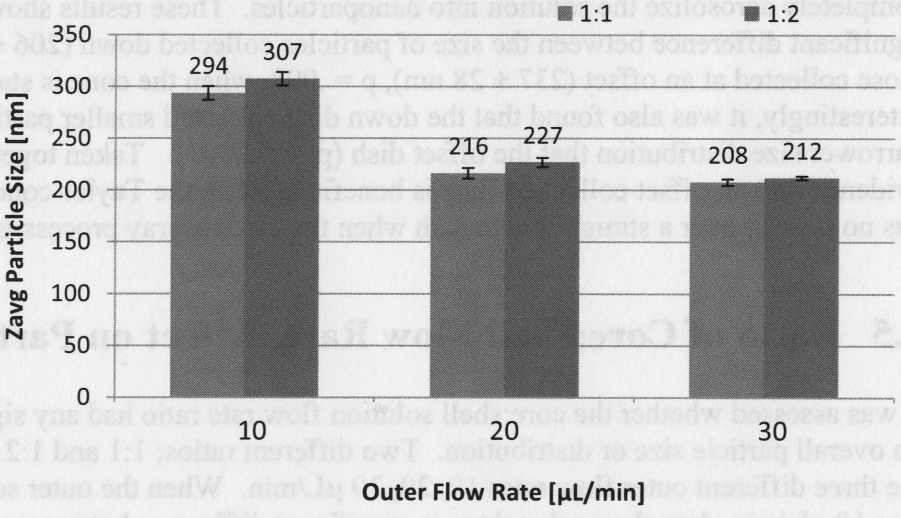



Figure 3.13: Comparison of 1:1 vs. 1:2 core:shell solution flow rates on particle size.

The difference between 1:1 and 1:2 for 10 μL/min was statistically significant, as was the difference between 1:1 and 1:2 for 20 μL/min. The difference in data between 1:1 and 1:2 for 30 μL/min was *just barely above* the criteria for significance. Overall, this data shows that flow rate ratio does play a role in affecting particle size. Moreover, Figure 3.13 shows a consistent trend of slightly larger particles created by a 1:2 ratio compared to 1:1 (core:shell) across all three shell solution rates (10, 20, 30).

## 3.6 Repeatability

Six independent electrosprays (n=6) were performed to determine if the parameters identified in previous experiments were consistent at reproducing small, narrowly dispersed sized particles. The results show that a mean particle size 217 ± 21 nm (PDI = .19 ± .05) can be repeatedly produced using the electrospray process.

| | | Experiment # | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| Scan 1 | Zavg | 215.60 | 180.90 | 241.00 | 206.40 | 208.20 | 242.90 |
| | PDI | 0.18 | 0.22 | 0.18 | 0.11 | 0.28 | 0.21 |
| Scan 2 | Zavg | 213.90 | 188.50 | 246.40 | 212.80 | 217.00 | 227.70 |
| | PDI | 0.14 | 0.23 | 0.20 | 0.14 | 0.25 | 0.20 |
| Average | Zavg | 215 | 185 | 244 | 210 | 213 | 235 |
| | PDI | 0.16 | 0.22 | 0.19 | 0.13 | 0.26 | 0.21 |
| Stand. Dev. | | 1 | 5 | 4 | 5 | 6 | 11 |

Table 3.8: Repeatability data

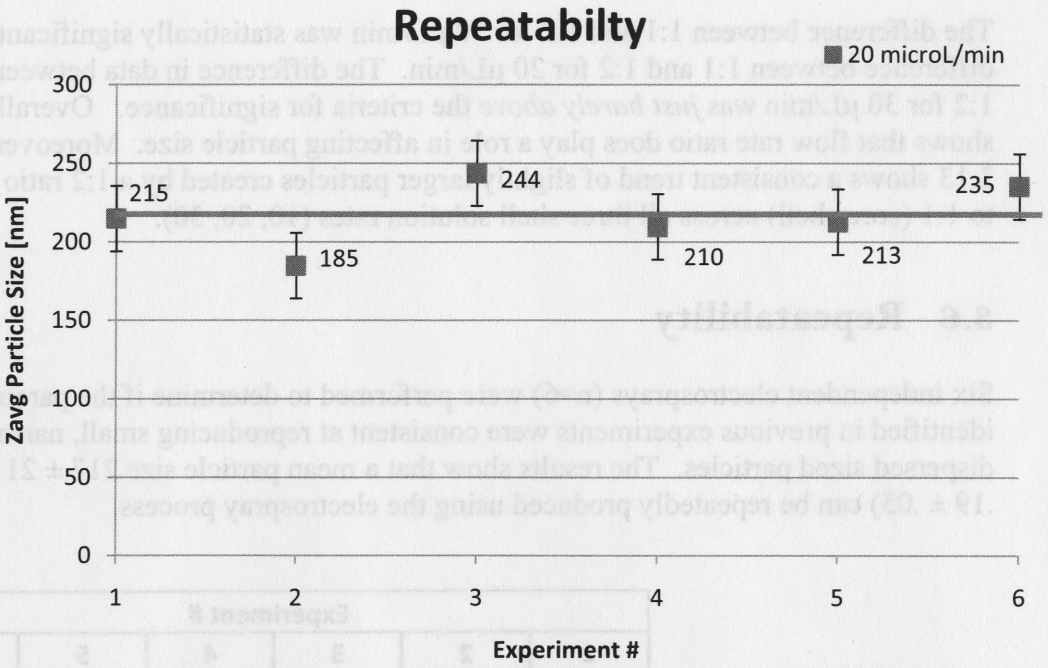



Figure 3.14: Zavg particle sizes from repeatability experiments. The mean (n=6) particle size is 217 ± 21 nm.

### 3.6.1 SEM Images

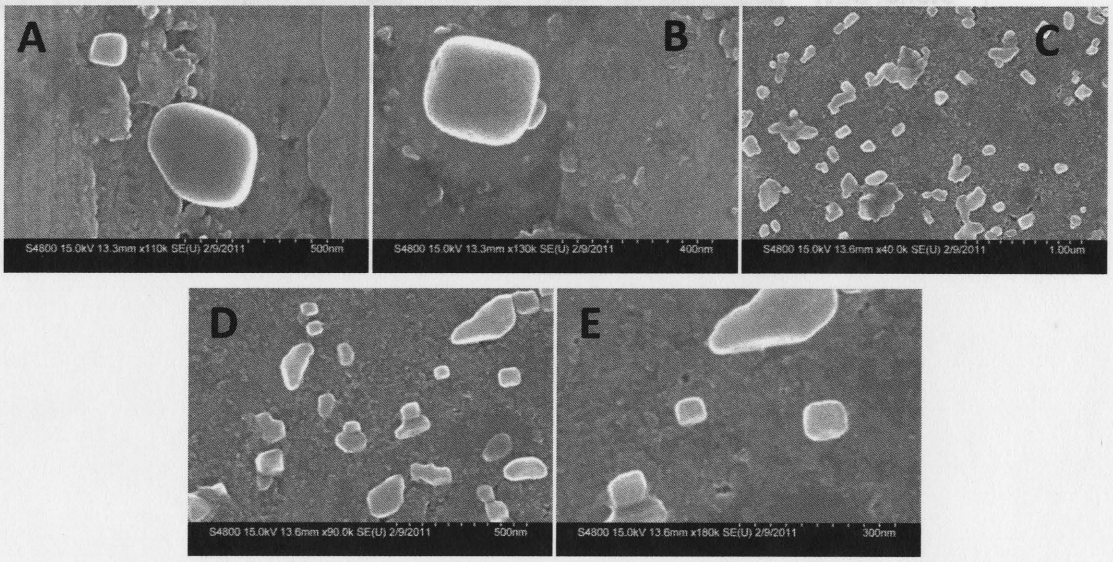



Figure 3.15: SEM images of coaxially electrosprayed cerasome particles

Based on images (A-E, Figure 3.15), the cerasomes collected on the aluminum peg during this experiment were in the range of 100-400 nm. Particle morphology appears to be an irregular or square-like shape, rather than the expected spherical shape. Also, particle density in the viewing area was low, compared to SEM images from other cerasome literature.

### 3.6.2 DLS Size Comparison with SEM

| | Flow rate = 30,27 (core,shell) | | | Voltage = 11.5 kV | |
|---|---|---|---|---|---|
| | | | | Range | |
| | Zavg | PDI | PDI width | low | high |
| | 251 | 0.297 | 136.8 | 114.2 | 387.8 |
| | 267.6 | 0.297 | 145.7 | 121.9 | 413.3 |
| | 246 | 0.327 | 140.6 | 105.4 | 386.6 |
| | 246.9 | 0.298 | 134.7 | 112.2 | 381.6 |
| Average | 253 | 0.30 | | 113.4 | 392.3 |
| Standard Dev. | 10 | 0.01 | | | |

Table 3.9: DLS size comparison with SEM

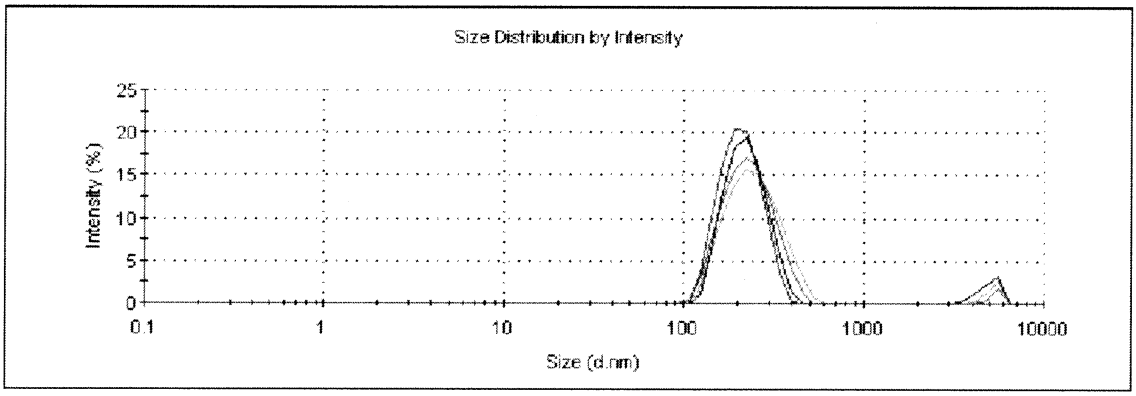



Figure 3.16: DLS results for SEM comparison

The particles collected in di-water during the simultaneous collection on SEM aluminum pegs were evaluated using DLS. The Zavg particle size was 253 ± 10 with PDI = .30 ± .01. Using PDI width data provided by the DLS measurement, a range of low-high values were calculated for each scan of the sample. An mean (n=4) of the low and high values shows the size range of cerasome particles in the solution to be approximately 100-400. This correlates precisely with what was observed in the SEM images.

## 3.7 Particle Stability

A two week stability test was performed to evaluate the effect of hydrated storage conditions (particles suspended in water, 4 °C) on both size and PDI. This effect was examined on particles created at two different flow rates, 20 and 40 µL/min. The total increase in particle size over two weeks was 42 nm and 50 nm, for the 20 and 40 µL/min samples respectively. For both samples tested, the PDI varied throughout the two week testing time, but neither increased nor decreased on a consistent basis. For the 20 µL/min sample, it was measured to be 0.22 (day 1), 0.19 (day 2), 0.17 (day 6), and then 0.20 (by day 14). For the 40 µL/min sample, it began at 0.13, went up to 0.18, slightly down to 0.17, then ended at 0.19 by day 14. Overall, neither sample had a substantial change in PDI over the course of two weeks.

| Flow Rate [µL/min] | | Day | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | 2 | | 6 | | 14 | |
| | | Zavg | PDI | Zavg | PDI | Zavg | PDI | Zavg | PDI |
| 20 | Scan 1 | 180.9 | 0.219 | 183.9 | 0.178 | 179.2 | 0.170 | 227 | 0.197 |
| | Scan 2 | 188.5 | 0.229 | 198.3 | 0.205 | 199.30 | 0.169 | 226.4 | 0.203 |
| | Average | 185 | 0.22 | 191 | 0.19 | 189 | 0.17 | 227 | 0.20 |
| | Stand. Dev. | 5 | 0.01 | 10 | 0.02 | 14 | 0.00 | 0 | 0.00 |
| 40 | Scan 1 | 185.6 | 0.117 | 192.1 | 0.157 | 207.2 | 0.173 | 241.4 | 0.184 |
| | Scan 2 | 201.3 | 0.142 | 207.9 | 0.205 | 218 | 0.168 | 245.2 | 0.199 |
| | Average | 193 | 0.13 | 200 | 0.18 | 213 | 0.17 | 243 | 0.19 |
| | Stand. Dev. | 11 | 0.02 | 11 | 0.03 | 8 | 0.00 | 3 | 0.01 |

Table 3.10: Particle stability in collection solution

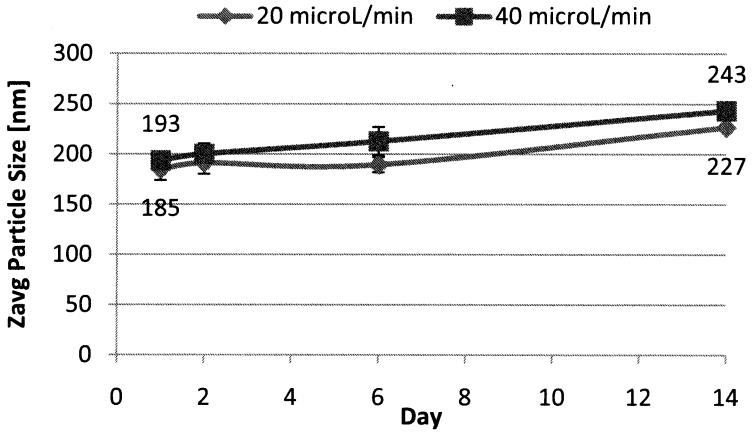



Figure 3.17: Particle stability in collection solution

# Chapter 4

# Discussion

The field of nanomedicine and drug delivery systems are in need of both a superior carrier vehicle and preparation technique than what is currently being used. Liposomes have demonstrated success in both the lab and clinic, and are currently the most widely used drug delivery vehicle. Despite wonderful biocompatibility and many great advantages, liposome suffer from an inherently weak membrane stability. Exposure to conditions of low (or high) pH, surfactants, or mechanical stress cause them to destabilize and consequently release their drug. Additionally, liposomes have a tendency to aggregate and fuse with other liposomes, which increases their size and facilitates clearance from the body. Both premature drug release and short circulation times limit the therapeutic effectiveness of liposomes. Recently, a novel organic-inorganic hybrid known as a "cerasome," for its siloxane surface network, has been developed. Due to the improved membrane strength, cerasomes have demonstrated superior morphological stability (against destabilization and aggregation) over liposomes. Additionally, cerasomes have a slower and more controlled drug release profile, which enhances their therapeutic effect.

Despite the creation of a superior liposome-like drug delivery vehicle, there remains a lack of an efficient and effective preparation technique. Laboratory methods, such as the film method, sol-gel/self assembly, reverse phase evaporation, freeze/thaw, etc. are commonly used to prepare liposomal drug vesicles. The problem with these methods is that they are multi-step, lengthy, and often complication processes that produce less than idea particles (i.e. polydisperse size distribution, low encapsulation efficiency, and low drug loading content). Recently, a one-step, facile technique known as coaxial electrospraying has emerged. Having demonstrated the ability to address and correct these issues makes electrospraying a very promising technique. While electrospraying has shown great success using standard liposome lipids and biodegradable polymer materials, it has never yet been used to create core-shell cerasome particles. The combination of using a more superior vesicle material with a more efficient preparation technique is an exciting new area of research. This study sets out to demonstrate that the coaxial electrospraying technique can successfully produce core-shell cerasome vesicles of the optimal size (100-300 nm) and distribution (PDI<0.2) for drug delivery applications.

Prior to beginning experiments, an extensive literature review uncovered many of the important parameters and their affect on the electrospraying process. Based on this

review, an experimental plan was devised. It was hypothesized that given a solution of constant concentration, conductivity, viscosity, and surface tension, both flow rate and voltage could be varied to tailor particle size. Specifically, there would be a particular voltage that would create an electric field of appropriate strength to aerosolize the solution. Once this was found, particle size could be reduced to within 100-300 nm by decreasing the flow rate. Upon experimentation, this hypothesis was found to be only true in part. There was a specific voltage that stabilized the Taylor cone (11 ± 1kV), but varying flow rate from 5-40 μL/min showed no significant affect on particle size. All flow rates within this range produced particles between 190-230 nm, with PDI < 0.2.

***Effect of Electrical Field Strength (Voltage)***

To determined the sole effect of voltage on particle size, all parameters were held constant. At a flow rate of 10 μL/min, it was found that 11 kV produced particles with the smallest size and distribution. Figure 3.3 shows photographs of the Taylor cone as it forms, stabilizes, and then destabilizes throughout the range of 7-15 kV. At 9 kV, the cone began to form. As voltage increased past 12 kV, the cone destabilized and particles became increasingly larger and more polydisperse. The most significant finding from this experiment was that the voltage that produced the best particles was also the one that stabilized the Taylor cone. Comparing visual images of the cone formation with the Zavg and PDI data, there is a clear relationship between a stable cone and high quality particles. This experiment solidified the importance of the voltage parameter in the electrospray process.

***Effect of Flow Rate***

Looking at the results of the previous experiments, it was questioned whether flow rate would have the same effect. To test this, voltage was held constant at 15 kV because this voltage does *not* create a stable Taylor cone. When 15 kV was used with a flow rate of 10 μL/min in the previous experiments, it produced large, polydisperse particles. This particular experiment would examine if a 15 kV electric field could be made to produce small, narrowly distributed particles solely by adjusting the flow rate. If the hypothesis that only one specific voltage would work for this particular solution, no amount of variation to flow rate could make 15 kV produce a stable Taylor cone. It was found that as flow rate was decreased from 10 to 6 μL/min, the electrospray process became slightly more stable, noted by a decrease in both particle size and PDI. Despite an improvement in particle quality, the Taylor cone never emerged as stable. The effect of changing flow rate (with constant voltage) did not have the same effect on improving particle quality as did changing voltage (with constant flow rate). This finding points towards voltage being the key factor in Taylor cone stability and thus particle size uniformity. Because of this data, a new experimentation was designed in an attempt to produce particles of ideal drug delivery size (200 nm) and distribution (<0.2) by varying flow rate, but this time using whatever voltage would create a stable Taylor cone for each flow rate.

***Effect of Flow Rate (with Stable Taylor Cone)***

It was decided to test the affect of flow rate on particle size and distribution when voltage

was adjusted to create a stable Taylor cone for each flow rate examined. Figure 3.6 shows photographs of the Taylor cone as voltage is adjusted between 8 and 12 kV. A well defined and stable cone emerges at 10 and recedes at 12 kV. Interestingly, this was the same for all three flow rates examined; 5, 20, 40 μL/min (photographs not shown for 5 and 40 μL/min). Figure 3.3, which showed the Taylor cone stability over the range of 7-15 kV using a flow rate = 10 μL/min, also exhibits a stable cone in this exact same range. It is thus concluded that for the particular cersasome solution properties and concentration being used, all flow rates between 5-40 μL/min require a voltage of 11 ± 1 kV to form a stable Taylor cone. This finding supports the hypothesis that there would be one specific voltage that permits a stable electrospray, but refutes the notion that flow rate could tailor particle size. As for the slight (±1 kV) variation in required voltage, it is believed that minor, unintended differences in the preparation of the cerasome solution slightly alter its physical properties. Factors such as precise concentration, hydrolyzation time, and time in storage may all contribute to the varying physical properties of the solution between experiments.

Identifying the voltage required to create a stable electrospray process is vital to the successful production of small, narrowly distributed particles. Once this required voltage value was established to be 11 ± 1 kV, it was possible to go forth and test the affects of varying flow rate on particle size. It was expected, based on literature, that varying flow would have a significant affect in controlling particle size. The findings from this study were surprising in that they did not support this. However, the goal of creating 200 nm sized particles (<0.2 PDI) was achieved regardless. All three flow rates (5, 20, and 40 μL/min) created particles in the range of 190-230 nm with less than 0.2 PDI. Table 3.4 shows the p-value significance between each of the three flow rates. A p-value of .046 between the 20 and 40 μL/min flow rates is the only one that shows a barely "significant" difference (95% confidence). Despite this, varying flow rate shows no form of a usable pattern for particle tailoring. Based on the PDI, 5 μL/min produced the most best particles (0.08 ±0.03), followed by 40 μL/min (0.14 ±0.01) and then 20 μL/min (0.16 ±0.03). Based on average and standard deviation of the Zavg size measurements, the same pattern was observed. 5 μL/min created the overall smallest particles. Taking average size, standard deviation, and PDI of the data collected into consideration, it is concluded that there is no *significant* size-related affect of varying the flow rate between 5 -40 μL/min, which represents the operable range for electrospraying cerasomes.

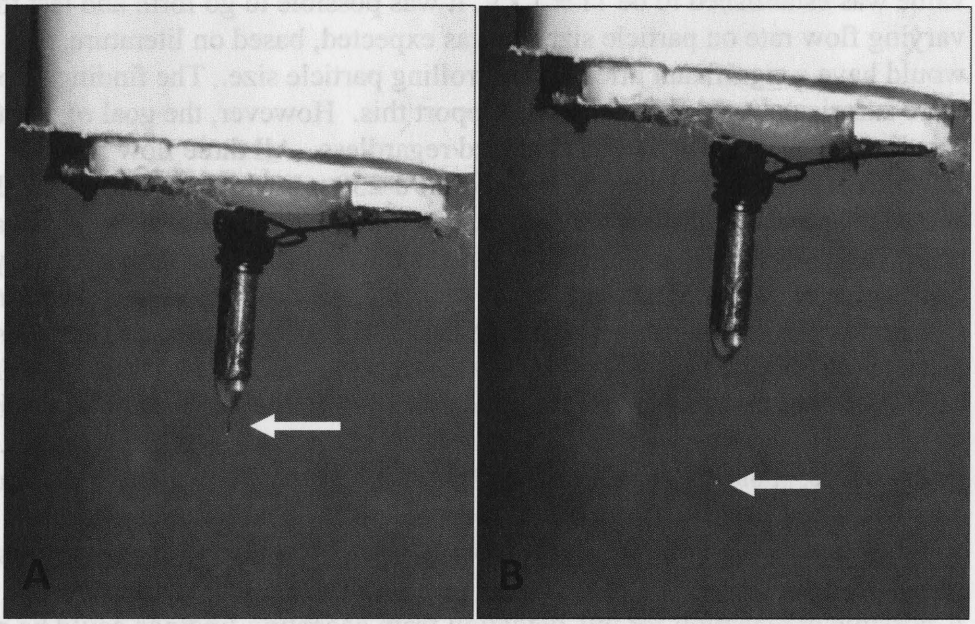
A possible explanation for this deviation from accepting findings could be as simple as the need for more data. Because of the overwhelming evidence that there should be a proportional relationship between the flow rate and size, it would be beneficial to re-visit these experiments in the future. A larger range of flow rates (beyond 5-40 μL/min) may bring forth a pattern not observed in these experiments.

Despite inconclusive results for the affect of flow rate on particle size, the goal of creating 200 nm sized particles with <0.2 PDI was accomplished using all three flow rates. 5 and 20 μL/min created the smallest particles with lowest PDI, and no *significant*

different between them. However, for reasons of increased speed of production (an important clinical feature), 20 μL/min was used as the flow rate of choice for subsequent experiments.

***Down vs. Offset Collection Dish***

The parameters necessary to create a stable cone have been established. Despite this, there was an unexplainable inconsistency between samples created using the exact same parameters. After careful examination of the Taylor cone during an entire spray, it was observed than even stable Taylor cones can exhibit brief, transient periods of instability (Figure 4.1). This unpredictable and unavoidable instability was believed to be responsible for inconsistencies in our samples. In an attempt to avoid collected large droplets from an unstable cone, the effects of an offset collection dish were explored. These experiments were conducted by using both a collection dish directly below the needles, and one slightly to the side. It was hypothesized that when an unstable cone was used, the down dish would collect larger particles with larger PDI than the offset dish. Additionally, under a stable cone, the offset would not be necessary.



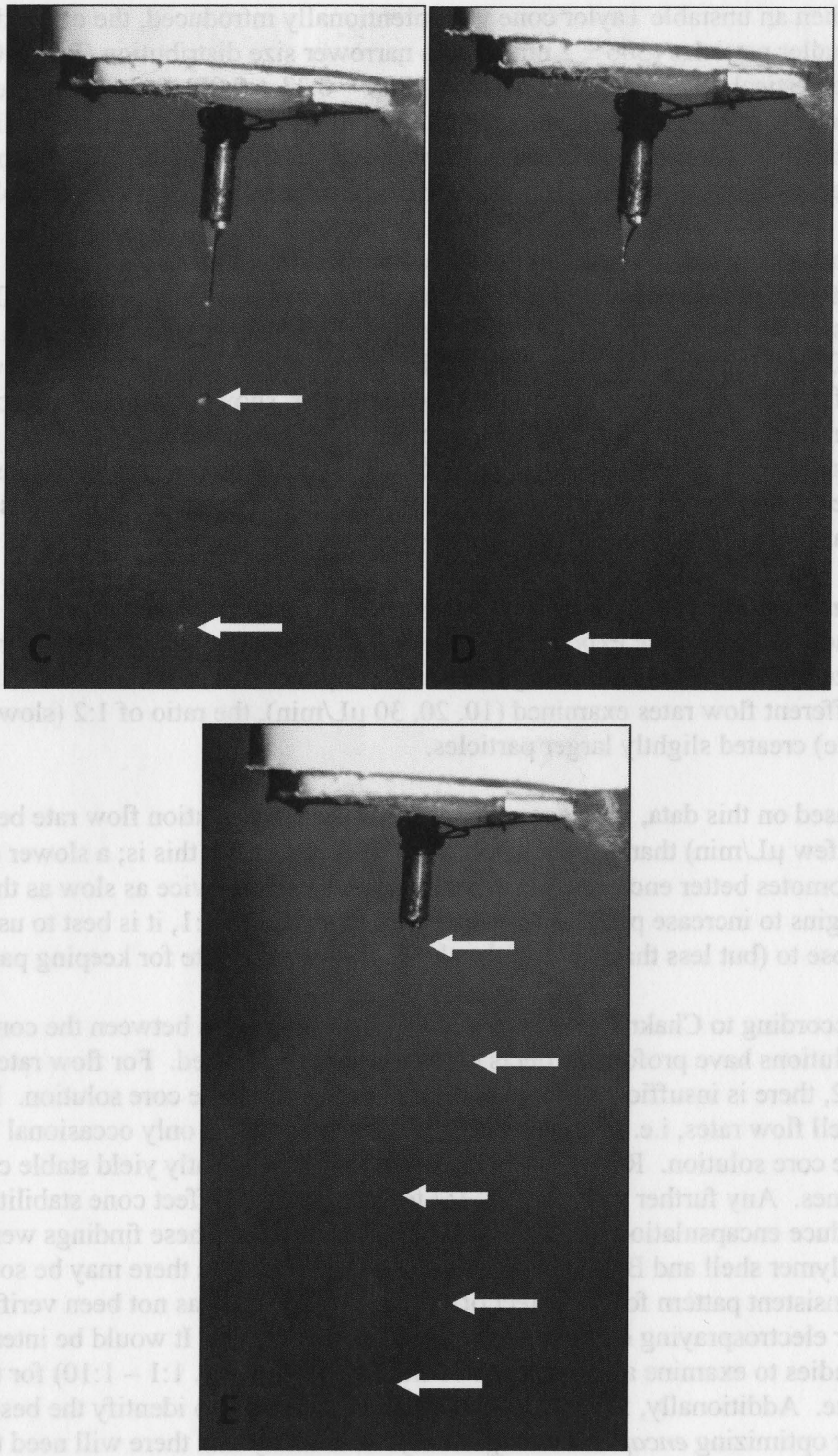



Figure 4.1 Unstable Taylor cones. A, B = 8 kV. C, D = 9 kV. E = 13 kV.

When an unstable Taylor cone was intentionally introduced, the offset dish collected both smaller particles (306 ± 2 nm) with a narrower size distribution (PDI = 0.25 ± 0.04) than the vertical down dish (338 ± 15 nm, PDI = 0.33 ± 0.07), as hypothesized. Statistical analysis reveals a significant ($p = 0.0047$) difference in the particles collected based on size (Zavg), but not significant difference ($p = 0.074$) based on PDI. These results show the benefit of using an offset when the electrospray process is not completely stable.

However, when a stable Taylor cone was used, there was no significant difference between the size of particles collected in the down vs. offset dish ($p = .098$). The down dish did, however, collect a smaller average size and standard deviation of particles (206±14 nm vs. 237±28 nm). In addition, the down dish had a much lower PDI than the offset dish ($p<.0001$). This data, taken together, shows that when the cone is stable, there is no benefit of using an offset collection dish. However, because brief periods of instability are both likely and unpredictable, the offset dish set-up is a useful safety mechanism to improve consistency between experiments. For this reason, a slight offset was implemented for all subsequent experiments.

***Ratio of Core:Shell Solution Flow Rate, Effect on Particle Size***

The results of these experiments showed that there is a significant difference between the size of particles created using a 1:1 and 1:2 (core:shell) flow rate ratio. For the three different flow rates examined (10, 20, 30 μL/min), the ratio of 1:2 (slower core solution rate) created slightly larger particles.

Based on this data, it is recommended that the core solution flow rate be only *slightly less* (a few μL/min) than the shell solution. This reason for this is; a slower core flow rate promotes better encapsulation. But, since a core rate twice as slow as the shell rate (1:2) begins to increase particle size compared to a ratio of 1:1, it is best to use a core flow rate close to (but less than) that of the shell solution flow rate for keeping particles small.

According to Chakroborty et al. [2009], flow rate ratios between the core and shell solutions have profound effects on the particles produced. For flow rate ratios less than 1:2, there is insufficient shell solution to encapsulate the core solution. For increased shell flow rates, i.e. ratios between 1:2 and 1:3, there is only occasional encapsulation of the core solution. Ratios between 1:3 and 1:6 consistently yield stable core/shell Taylor cones. Any further increase, i.e. 1:7 to 1:10 does not affect cone stability, but begins to reduce encapsulation efficiency of the core solution. These findings were made using polymer shell and BSA core electrosprun fibers. While there may be some universally consistent pattern for the affect of flow rate ratio, this has not been verified specifically for electrospraying other materials, such as cerasomes. It would be interesting for future studies to examine a larger range of flow rate ratios (i.e. 1:1 – 1:10) for their affect on size. Additionally, further experimentation is needed to identify the best flow rate ratio for optimizing *encapsulation efficiency.* It is likely that there will need to be a compromise between the best ratio for reducing size and that for increasing encapsulation efficiency.

***Repeatability***

Using the parameters identified through experimentation, cerasome particles of average size 217 ± 17 nm (PDI=.19 ± .05) can be repeatedly produced using the electrospray process. This size is optimal for both body clearance evasion and tumor vasculature penetration. Repeatability is an important factor if the coaxial electrospray method for core-shell particle production is to ever have widespread laboratory or clinical acceptance.

SEM images were taken to both observe the particle morphology and determine the accuracy of DLS size and distribution measurements. The sizes measured for one particular sample under DLS (Zavg: 253 ± 10 nm, PDI = 0.30 ± .01) were confirmed by SEM images. Both DLS and SEM show a distribution of approximately 100-400 nm. The morphology, however, was not as expected. Rather than smooth, spherical particles, the cerasomes exhibit a flattened, irregular and often square-like shape. This could be a result of core solution (di-water) evaporation or leakage from the vesicles prior to SEM anaylsis. As such, the particles may deflate and flatten out on the aluminum peg surface. Another possible explanation is that vacuum conditions and the platinum sputter coating flattened the particles. To fully understand the reason for this observed morphology, further investigation will be necessary,

The major difference between SEM images of this study and others referenced is the density of particles in the viewing area. In our images, there is a very low density of particles. This could be because the aluminum peg was not grounded during the collection process. It was held suspended above the collection dish, so that particles could passively land on the surface as they moved toward the grounded aluminum foil. Because it the peg was not grounded, however, it is suspected that the particles moved around the peg to land in the dish. Only a small fraction of the particles were collected on the surface.

***Particle Stability***

After creating cerasome particles, there was often a short wait period before DLS access became available. This wait ranged from 1-7 days. During this time, particle solutions were kept suspended in their collected, hydrated state in 4°C refrigeration. To examine the affect of this storage time on particle size, a two week stability test was performed. Two particle samples were created, one using 20 μL/min flow rate, the other 40 μL/min. The results show that over two weeks, the particle size increased by 42 and 50 nm, respectively. The solution prepared at 20 μL/min had a decrease in PDI from 0.22 to 0.20 at two weeks. The solution prepared at 40 μL/min had an increase in PDI from 0.13 to 0.19 at two weeks. This conflicting data points toward an insignificant change in PDI over the two weeks. Because size increased independent of size distribution (PDI), we suggest that it is due to absorption of water, and thus swelling of the particles. Since the particle are loaded with di-water and also suspended in a hydrated storage condition of di-water, it is plausible that absorption has caused the gradual size increase. It is not

believed that the size increase is due to aggregation or fusion of particles. If this were the case, we would expect to see a large increase in size distribution (PDI) as some particles would be doubling or tripling in size as others remain unchanged. An unchanged PDI makes this explanation unlikely. The information gathered in this experiment is significant because it provides a linear reference to how much the particles can be expected to expand at any given time (up to 14 days). By fitting a trend line to the data, it provides a way to standardize the size measurement of particles that are obtained after different lengths of time in storage. See Figure 4.2.

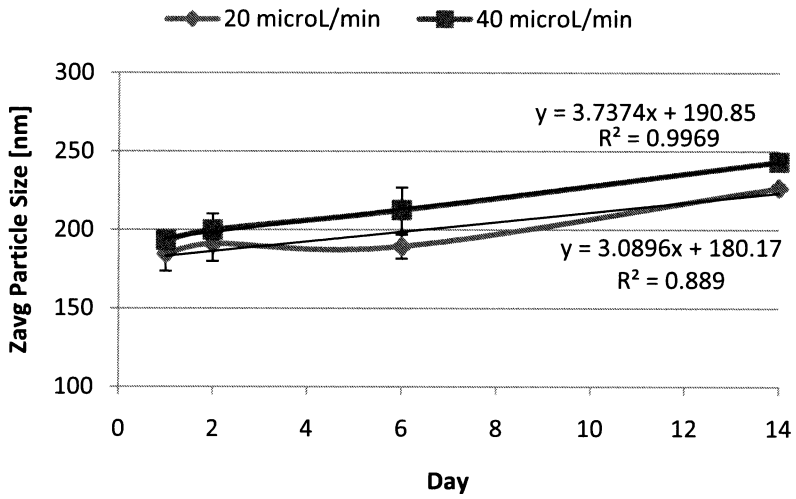



Figure 4.2: 14 day particle stability (with linear trend lines)

Future work should run this storage stability experiment for a longer period of time to see if the pattern continues or eventually plateaus. If the size increase is due to swelling, there should be a point where the particles reach maximum expansion and plateau. A long term experiment would also provide important information on how long cerasomes remain stable in storage conditions, or at what point they being to aggregate or destabilize. This information has important clinical implications.

### *Additional Findings*

The following discussion is based on the results of experiments not mentioned in the results section of this study.

*Solution Preparation*

Before electrospraying and collection particles, the cerasome lipids were hydrolyzed in pH3 ethanol for 24 hours, then given an additional 24 hours incubation period to allow for a complete surface siloxane network formation. When this 24 hour time period was not permitted, and electrosprays were conducted immediately after hydrolyzation, the particles produced had very large Zavg size and size distribution (PDI) measurements. The DLS "Expert Advice" feature indicates that the solution is not stable, and that particles may be aggregating. This makes sense, given that the additional wait time is for the siloxane network to completely form, which then prevents aggregation. Figure 1.10 shows support for why post-processing incubation for an additional 24 hours helps to strengthen the vesicle against destabilization. Furthermore, Cao et al. [2010] report that freshly prepared cerasomes were stable in alkaline condition (supported by the zeta potential chart), but were sensitive to acid. The inorganic siloxane network was not well developed on the surface of newly prepared cerasomes. But, after 24 hours, cerasomes showed remarkable morphological resistance toward both acidic and alkaline solution. These findings in literature support our results.

*Effect of Radial Distance on Particle Size*

Radial distance refers to the distance extending outward in any direction from a point directly below the needles. Particles ejected from the needle tip travel in a conical shape towards the ground. Although no previous studies mention radial distance as a factor, it was hypothesized, based on results showing that an offset was beneficial, that if particles being sprayed are polydisperse, they will spray radially according to their size and mass. Specifically, large particles will spray closer to directly down, whereas the smaller and lighter particles will travel further from the needles. If this were true, it would then be possible to collect the desired sized particles by placing the collection dish a certain radial distance from the needle. The exact distance would be determined through experimentation, and would also likely vary with other parameters such as flow rate and or voltage.

To test this hypothesis, a target dish was created using four different size tissue culture dishes aligned concentrically (Figure 4.3). This created thin circular zones to collect particles traveling different radial distances. The center zone covered radial distance 0-0.5 cm. The second zone covered radial distance from 0.5 to 2.5 cm. The third zone covered distance from 2.5 cm to 4 cm. The outermost zone collected particles traveling 4 cm to 7 cm. Two independent trials were conducted using the target dish collection set-up. In both cases, there was no evidence to support the hypothesis of radial distance effecting particle size. The particles collected in the zones had varying sizes and PDIs with no particular consistency. Additionally, the two trials showed no common result

trends. This data was not beneficial towards the progression of this study, and as such, was omitted from the results section.

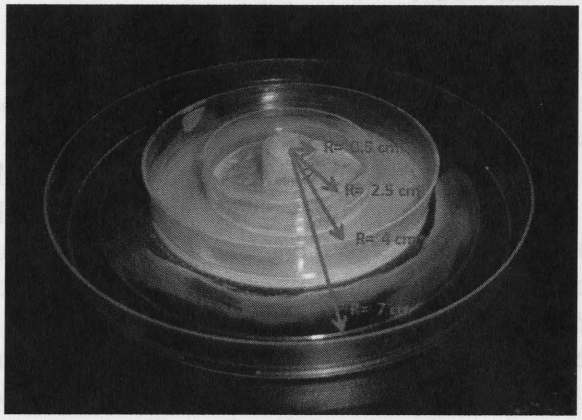



Figure 4.3: Target dish

*Doxorubucin Encapsulation*

Initial experiments for encapsulating doxorubicin into cerasome vesicles using the coaxial electrospraying technique are underway. To date, 1.67 mM doxorubicin hydrochloride in water has been used as a core solution to load cerasome vesicles. It has been shown that dox-loading can be achieved using the same parameters as those used to create di-water loaded vesicles; core-shell flow rate: 18-20 μL/min., voltage 11 ± 1 kV (Figure 4.2). In addition, dox-loading does not appreciably alter vesicle size (220 ±13 nm, PDI = 0.3 ± 0.02) compared to di-water-loaded cerasomes (217 ± 21 nm, PDI = .19 ± 0.04).

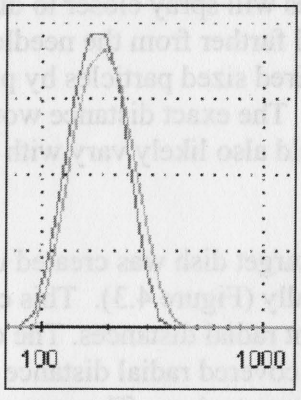



Figure 4.4: DLS measurement of doxorubicin loaded cerasomes

It should be noted that concentration of doxorubicin dissolved in water for this experiment was not at maximum solubility. When future experiments measure and try to improve drug loading content, solubility will become an important factor.

## 4.1 Future Work

This study was successful in accomplishing both aims laid out in section 1.1 – *Specific Aims*. Briefly, these were to (1) demonstrate that the electrospraying technique is capable of producing cerasome vesicles, and (2) identify the parameters required to produce particles of optimal size (100-300 nm) and distribution (PDI<0.2) to be effective drug delivery vehicles. Both of these aims were met, and yet the work of this study is only laying the groundwork for the future of electrospraying cerasomes. Additional work that can build upon this study include, but is not limited to;

- Using cryo-SEM (or TEM) to visualize the core-shell structure of the cerasome vesicles. Our study used SEM to observe overall morphology and size distribution, but visual evidence of the core-shell structure is also needed.
- Extending the storage stability experiment for a longer period of time.
- Alter the cerasome-forming organotrialoxysilane lipid concentration to less than and greater than 15 mg/mL while examining the effect on electrospray parameters and particle size (or distribution)
- Encapsulate doxorubicin drug into the core of cerasome particles.
    - Verify that drug loading does not affect particle size
    - Visualize morphology and confirm size distribution under SEM
    - Use contrast agent to visualize drug encapsulation in the core (under cryo-SEM or TEM)
    - Measure encapsulation efficiency (E.E.)
        - Alter parameters (i.e. flow rate ratio) to improve E.E.
        - Measure drug loading content
        - Improve drug:lipid ratio by exploiting solubility of the drug. If max solubility in water is not enough, try DMSO. Doxorubicin is more soluble in DMSO (100 mg/mL) than water (10 mg/mL).
    - Show the drug release profile of doxorubicin loaded cerasomes under various pH and surfactant (TX-100, ethanol) conditions.
        - Adjust lipid concentration to improve membrane permeability (i.e. drug release profile)
        - Include membrane destabilizing modalities to create "trigger-able" drug release

## 4.2 Acknowledgments

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# Appendix

## A. Liposomal drugs

### A.1 FDA-approved liposomal drugs

| Drug name and company | Indications/Target | Reference |
|---|---|---|
| Marqib-Vincristine sulfate liposomes injection: Vincristine-encapsulated liposomes in a lipid bilayer of sphingomyelin (Hana Biosciences) | Treatment of adult patients with acute lymphoblastic leukemia | 245 |
| DaunoXome: Daunorubicin citrate-liposome injection (NeXstar Pharmaceuticals) | Advanced HIV-associated Kaposi's sarcoma for first-line use | 246 |
| AmBisome®: Lipid-based formulations, including liposomal amphotericin B (Fujisawa Healthcare) | Treatment of fungal infection and visceral leishmaniasis | 247,248 |
| Doxil®, caylex: PEGylated liposomal loaded with doxorubicin (PLD) (Ben Venue Laboratories for Johnson & Johnson) | Treatment of metastatic breast cancer, advanced ovarian cancer, multiple myeloma, and AIDS-related Kaposi's sarcoma | 249–252 |
| Amphocil: Lipid form of amphotericin B stabilized with cholesteryl sulfate (Samaritan Pharmaceuticals) | Amphocil binds to lipoproteins and ergosterol in cell membranes of infecting fungi; also indicated for the treatment of invasive aspergillosis and leishmaniasis | 253,254 |
| ABELCET®: Amphotericin B lipid complexed with two phospholipids (DMPC:DMPG, 7:3 molar ratio) in a 1:1 drug-to-lipid molar ratio (The Liposome Company) | ABELCET® consists of amphotericin B, a polyene, antifungal antibiotic for invasive fungal treatment | 255 |
| Depocyt™: Liposomal cytarabine or liposomal Ara-C are antimetabolite cytarabine, encapsulated into multivesicular lipid-based particles (Enzon Pharmaceuticals) | DepoCyt is a sustained release formulation of the chemotherapeutic agent cytarabine, used for the treatment of patients with lymphomatous meningitis | 256 |

Chart reproduced from Puri et al. [2009]

## A.2 Liposomal drugs in clinical trials

| Drug name and company | Treatment | Status | Reference |
|---|---|---|---|
| Liposomal-annamycin semi-synthetic doxorubicin analog: Annamycin intercalates into DNA and inhibits topoisomerase II (Aronex Pharmaceuticals) | Acute myeloid and lymphoid leukemia (drug-resistant tumors) | Phase I/II | 257,258 |
| Lipoplatin: A new cisplatin-encapsulated liposome composed of DPPG, soy phosphatidylcholine (SPC-3), cholesterol, and mPEG2000-DSPE, designed to reduce cisplatin toxicities without reducing efficacy (HBio) | Carcinoma of the head and neck, pancreatic cancer, and continuing in advanced breast cancer and gastrointestinal cancers | Phase II clinical trial and one phase III non-inferiority clinical study | 259,260 |
| NX 211 (liposomal lurtotecan): Liposomal formulation of the lurtotecan a topoisomerase I inhibitor (Gilead and Glaxo) | Advanced or recurrent ovarian epithelial cancer | Phase II | 261,262 |
| Liposomal vincristine (ONCO-TCS™): Vincristine encapsulated in liposomes composed of sphingomyelin and cholesterol (INEX Pharmaceuticals) | Treatment of relapsed aggressive non-Hodgkin's lymphoma and other cancers | Pivotal phase II/III | 245,263–265 |
| Doxorubicin HCL liposome for injection: Non-PEGylated liposomal doxorubicin Myocet made by Enzon Pharmaceticals for Cephelon in Europe and for Sopherion Therapeutics in the United States and Canada | Treatment for patients with stage II or III invasive breast cancer and with tests showing an overexpressing of human epidermal growth factor receptor 2; Myocet® is approved in Europe and Canada for treatment of metastatic breast cancer | Pivotal phase III global registrational trial for treatment of HER2-positive metastatic breast cancer | 266,267 |
| VentusTM: Prostaglandin E1 liposomes (The Liposome Company) | Pretreatment of acute respiratory distress syndrome | Phase III clinical trial | 268 |
| ThermoDoxTM: Lysolipid thermally sensitive liposomes are heat-activated liposomal encapsulation of doxorubicin (Celsion Corporation) | ThermoDoxTM can be used as treatment for hepatocellular carcinoma (primary liver cancer) and recurrent chest wall breast cancer | Phase III | 269 |
| Protein-stabilized liposome encapsulation of active drug Docetaxel (ATI-1123): Liposome product made in a single step and encapsulates Docetaxel a clinically well-established anti-mitotic chemotherapy medication (Azaya) | Docetaxel (trade name Taxotere) is used mainly for the treatment of breast, ovarian, and non-small cell lung cancer | Pre-clinical studies to FDA phase I clinical trial | 270 |

Chart reproduced from Puri et al. [2009]

## B. Effect of Flow Rate (with Stable Taylor Cone)

| | | Experiment # | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | | | | 2 | | | | 3 | | | | | |
| Flow Rate (outer) | | Scan 1 | Scan 2 | Average | Stand. Dev | Scan 1 | Scan 2 | Average | Stand. Dev | Scan 1 | Scan 2 | Average | Stand. Dev | Average | Stand. De |
| 5 | Zavg | 197.50 | 205.30 | 201.40 | 5.52 | 206.30 | 209.20 | 207.75 | 2.05 | 211.70 | 214.80 | 213.25 | 2.19 | **207.47** | 5.93 |
| | PDI | 0.13 | 0.06 | 0.09 | | 0.05 | 0.09 | 0.07 | | 0.13 | 0.11 | 0.12 | | **0.08** | 0.03 |
| 20 | Zavg | 215.6 | 213.9 | 214.75 | 1.20 | 206.4 | 212.8 | 209.60 | 4.53 | 241 | 246.4 | 243.70 | 3.82 | **222.68** | 18.38 |
| | PDI | 0.18 | 0.139 | 0.16 | | 0.114 | 0.142 | 0.13 | | 0.182 | 0.196 | 0.19 | | **0.16** | 0.03 |
| 40 | Zavg | 185.6 | 201.3 | 193.45 | 11.10 | 221 | 223.7 | 222.35 | 1.91 | 161.3 | 177.4 | 169.35 | 11.38 | **195.05** | 26.54 |
| | PDI | 0.117 | 0.142 | 0.13 | | 0.14 | 0.139 | 0.14 | | 0.154 | 0.142 | 0.15 | | **0.14** | 0.01 |

Table 3.3: Effect of varying flow rate (with Stable Taylor cone). Voltage = 11 ± 1kV for all flow rates tested.